THE BEHAVIOURAL PHARMACOLOGY OF MEPHEDRONE ("BATH SALTS") AND ITS EFFECTS ON ANXIETY-RELATED BEHAVIOURS IN MALE AND FEMALE HOODED RATS

A thesis submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy in Psychology

By

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2016

Acknowledgements

First and foremost I would like to thank my supervisor, Professor Rob Hughes, for his unwavering support and guidance over the past few years. Rob truly is a paragon of academic supervision; I could not have asked for more or better in a mentor, and it is largely due to his involvement that this project has gone as smoothly as it has.

Thanks also to Juan Canales and Andrew Watson for their early involvement and advice, and also to Anthony McLean for coming on board at such a late stage.

A big thank you to the Animal Facility staff, Neroli, Silvana, and Kate. You guys are the unsung heroes of all this research, without whom none of this could happen. Thank you for all the advice and assistance over the years, I enjoyed working with you all.

Finally, thank you to my family, friends, housemates, and colleagues, who have supported me and put up with me throughout this process. Particular thanks go to Jono Hackney, my long-suffering friend, who has shared this journey with me from day one. Thanks also to Wade Stent for his advice and feedback on final drafts.

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Abstract

The current generation of designer drugs of abuse are synthetic analogues of the stimulant cathinone. Popularly known as 'bath salts', these drugs are marketed as safer (and often legal) alternatives to known psychoactive drugs like methamphetamine, cocaine, and ecstasy. There is emerging literature on the pharmacology and toxicology of these 'bath salts', though to date few scientific studies have specifically examined the behavioural sequelae. The current study therefore sought to assess some of the behavioural effects of mephedrone, one of the most common primary active ingredients of 'bath salts'. Male and female hooded rats were used in three experiments examining anxiety-like behaviours in a number of established behavioural testing paradigms. Experiment 1 examined the dose-response relationship and found that mephedrone produced more anxiety-like behaviours as dose increased, though interestingly only for female rats (males instead displayed an inverted 'U' shaped dose-response curve). A large number of stereotyped behaviours were observed at high doses so this was examined in Experiment 2. It was found that as mephedrone dose increased, so too did the frequency of stereotyped behaviours, though this finding also differed across the sexes in its presentation (males tended to exhibit more non-ambulatory behaviours such as head swaying whereas females tended to display more locomotor stereotypies). Experiment 3 examined the adolescent behavioural teratology of mephedrone by chronically treating rats during adolescence, and then later testing them in adulthood. Results showed that adolescent mephedrone use increased anxiety-like behaviours later in life, though there was some evidence that this effect could be mitigated by environmental enrichment. Taken together, the findings of this study suggest that mephedrone is a powerful anxiogenic compound capable of producing behavioural changes following both acute and chronic administration. Of particular interest and concern is the different presentation of effects noted in male and female subjects.

1.0 General Introduction

The use and abuse of psychoactive compounds is a significant and all-to familiar problem for communities and governments around the world today. New 'designer drugs' produced to mimic the effects of known illicit substances are created at an alarming rate, and often legislative bodies, not to mention medical professionals, are left scrambling to 'catch up' after the fact. This 'cat and mouse' of drug creation and sale, constantly preceding legislation and understanding the known effects leads to a situation where unsuspecting people can purchase and consume substances, often legally, under the impression that they are well-tested and safe; an impression that is often far from reality. The so-called 'legal high' industry has evolved from the development of synthetic derivatives of fentanyl in the early 1980s, phenethylamines in the late 1980s, tryptamines in the 1990s, piperazines in the 2000s, to the current trend of synthesizing cathinone derivatives (Brandt, Freeman, Summale, Measham, & Cole, 2010; Coppola & Mondola, 2012a). These synthetic cathinones, commonly referred to as 'bath salts', are molecular analogues of amphetamine and have been said to possess the worst characteristics of methamphetamine, cocaine, lysergic acid diethylamide (LSD), phencyclidine (PCP, or 'angel dust'), and methylenedioxymethamphetamine (MDMA, or 'ecstasy') (Ross, Watson, & Goldberger, 2011). Bath salts are sometimes referred to as psychoactive bath salts (PABS) in order to differentiate them from the legitimate bathing products (magnesium sulphate crystals or 'Epsom salts' dissolved in bath water for cosmetic purposes; i.e., to improve cleaning, soften the skin, or for their aroma) whose name they have inaccurately taken (Browning, 1999; Ross et al., 2011). They describe a family of drugs whose active ingredients are one of several synthetically derived cathinone analogues, typically 3,4-methylenedioxypyrovalerone (MDPV) or 4-methylmethcathinone (mephedrone) (ACMD, 2010; Harvey & Baker, 2015; Winder, Stern, & Hosanagar, 2013; Zawilska, 2014).

This paper will examine the current literature on 'bath salts' with a specific emphasis on mephedrone, the variety thought most common in European countries and in New Zealand (Brandt et al., 2010; Farrier, 2010; Wood & Dargan, 2012). A note on terminology: the term 'bath salts' (the origins of which are explained in the next section) is popularly used to individually describe mephedrone, MDPV, methylone, and a number of other synthetic cathinone derivatives. For the purposes of this paper, the term 'bath salts' may be used synonymously with 'synthetic cathinones' (i.e., the *class* of drug that contains mephedrone, MDPV, and others). The individual drug's name will be used when referring to a specific compound (e.g., 'mephedrone').

1.1 Origin of "Bath Salts"

Cathinone is a naturally present alkaloid found in the Khat plant (*Catha edulis*), a leafy evergreen shrub native to several regions of Africa and the Arabian peninsula (Cameron, Kolanos, Vekariya, De Felice, & Glennon, 2013; McClean, Anspikian, & Tsuang, 2012). Locally the leaves of the khat shrub have traditionally been chewed or boiled in a tea to produce stimulation and a state of euphoria (Ali et al., 2010; Coppola & Mondola, 2012a; Kalix, 1986). Chewing the leaves for approximately one hour produces psychotropic effects that last for around 3 hours thereafter (Kalix, 1996). Cathinone exerts its effects on the brain by releasing catecholamines from pre-synaptic storage sites in the central and peripheral nervous system (Kalix, 1986), and it is also thought that it may act as a monoamine oxidase inhibitor, preventing the breakdown of monoamine neurotransmitters at the synapse and thus increasing their availability (Nencini, Amiconi, Befani, Abdullahi, & Anania, 1984). The World Health Organisation (WHO) classifies khat as a 'drug of abuse' (Nutt, King, Saulsbury, & Blakemore, 2007), and cathinone has 'Schedule 1' drug status under the Convention on Psychotropic Substances (INCB, 2015).

The first synthetic cathinones were methcathinone and mephedrone, produced as early as the late 1920s. However, with few (if any) therapeutic applications, and serious side effects noted, these drugs fell out of favour and common use for decades. It was only in the 2000's that they re-emerged as designer drugs of abuse, and became popular in the 'legal high' arena as recreational/party drugs (Coppola & Mondola, 2012b; Karila, Megarbane, Cottencin, & Lejoyeux, 2015). More recently, the first synthetic cathinone to come to the attention of European authorities was mephedrone, in 2007 (German, Fleckenstein, & Hanson, 2014; Karila et al., 2015; McClean et al., 2012). In the following years mephedrone, MDPV, and other synthetic cathinones received a surge of popularity in Europe and the USA (Dargan, Sedefov, Gallegos, & Wood, 2011; McClean et al., 2012; Wieland, Halter, & Levine, 2012). By 2010 mephedrone had been detected in 28 European countries (Karila et al., 2015) and a number of international governments were considering legislation to ban its use (ACMD, 2010).

The delay in implementing controls for these drugs was likely due in no small part to the tactics used by the developers: the drugs were marketed online and sold in head shops, petrol stations, and conveniences stores as 'bath salts' and labelled "not for human consumption" in an attempt to circumvent federal drug-analogue laws (Goshgarian, Dawn, & Caplan, 2011). Other surreptitious attempts to avoid regulatory scrutiny saw them sold as other innocuous substances such as 'plant food' and 'fertiliser' (Borek & Holstege, 2012). These false bath salts were similar in appearance to genuine bath salts (fine white powder or crystals) and were sold in small individual sachets with brand names like 'Ivory Wave', 'White Lighting', 'Vanilla Sky', and 'Cloud 9'. Their ready availability (particularly over the internet), low price, and the fact that they were marketed as safe and legal alternatives to other drugs of abuse led to a surge in their popularity, particularly among young people (Brandt et al., 2010; Dargan, Albert, & Wood, 2010; Loi et al., 2015). Despite subsequent

legislation rendering the spurious labelling redundant, the term ‘bath salts’ has remained, becoming synonymous with this growing group of synthetic cathinone derivatives.

1.2 Pharmacokinetics

Bath salts are generally sold as a white crystalline powder and can be taken intranasally (snorting), orally, intravenously, intramuscularly, or rectally (Dargan et al., 2011). In a 2011 survey of nearly 1000 mephedrone users in the UK, the most popular method of administration was snorting (65.9%), though the authors noted that many of these users reported significant nasal irritation, which in turn led to a change from nasal to oral administration (Winstock et al., 2011). Additionally, oral users have reported that the powder has an unpleasant taste, so oral administration is usually achieved by dissolving the powder in water, ‘pressing’ the powder into tablets, or wrapping the powder in cigarette paper and swallowing (‘bombing’) (Dargan et al., 2011). The white powder is easily soluble in water making injection another viable administration route. Bath salts appear to be psychoactively effective at doses as low as 3-5mg, with typical doses being between 5 and 250mg per session (Dargan et al., 2011; Pasties, 2010; Ross et al., 2011). Users report initially using lower doses (50-75mg) then increasing quickly to doses in the hundreds of milligrams (Newcombe, 2009). In the UK clubbers’ survey, 22.3% of participants reported using more than 1 gram in a typical session (Winstock et al., 2011). Intravenous mephedrone use is associated with higher doses, more frequent use, and an elevated psychiatric symptom profile (Kapitany-Foveny et al., 2015). The onset of action depends on the method of administration and is usually between 15 to 45 minutes when taken orally, and within minutes for nasal insufflation, with peak effects after 30 minutes (McClean et al., 2012). Despite mimicking the effects of known stimulants bath salts are not usually detected in regular urine analysis (Winder et al., 2013). Successful detection in hair or urine samples requires gas chromatography-mass spectrometry (Strano-Rossi, Cadwallader, de la Torre, & Botre, 2010).

1.3 Pharmacodynamics

1.3.1 Desired effects. Synthetic cathinones are structurally similar to amphetamine, MDMA, and cocaine and thus produce stimulant and sympathomimetic effects comparable to these compounds (ACMD, 2010; Gatch, Rutledge, & Forster, 2015; Varner et al., 2013; Wieland et al., 2012; Winstock, Marsden, & Mitcheson, 2010). The desired effects for which they are taken include euphoria, elevated mood, improved mental function, increased sociability, enhanced appreciation of music, and mild sexual stimulation (ACMD, 2010; Dargan et al., 2011; Winstock et al., 2011). Users report that the 'high' associated with mephedrone use is better and longer-lasting than that of cocaine (Winstock et al., 2011). The desired effects are said to last for around 2 to 4 hours, while some secondary effects such as mild stimulation, hypertension, and tachycardia can last for over 8 hours (Dargan et al., 2011; DEA, 2011; Martinez-Clemente et al., 2013).

1.3.2. Side effects. While the scientific literature is still relatively sparse on the side effects of using bath salts there is a significant body of information comprised of case studies, emergency room reports, and calls to poison control centres. There are documented cases of bath salts users experiencing severe paranoid psychosis (Antonowicz, Metzger, & Ramanujam, 2011; Goshgarian et al., 2011; Kasick, McKnight, & Klisovic, 2012; McClean et al., 2012; Striebel & Pierre, 2011), hallucinatory delirium (Penders & Gestring, 2011), extreme agitation and violent behaviour (Penders, Gestring, & Vilensky, 2012), confusion, syncope, and tachycardia (Smith, Cardile, & Miller, 2011; Wood et al., 2010), serotonin syndrome (Mugele, Nanagas, & Tormoehlen, 2012), and hyperthermia leading to multiorgan system failure and death (Borek & Holstege, 2012). Table 1 presents a list of possible effects as described in the current literature.

Table 1.
Physical and Behavioural Effects of Bath Salts (Ross et al., 2011)

Physical Effects	**Behavioral and Mental-Status Effects**
Tachycardia	Panic attacks
Hypertension	Anxiety
Vasoconstriction	Agitation
Arrhythmias	Paranoia
Hyperthermia	Hallucinations
Sweating	Psychosis
Mydriasis	Aggressive behavior
Muscle tremor and spasms	Violent behavior
Seizures	Self-destructive behavior
Stroke	Self-mutilation
Cerebral edema	Suicidal ideation
Respiratory distress	Insomnia
Cardiovascular collapse	Anorexia
Myocardial infarction	Depression
Death	

1.3.3 Mode of action. Synthetic cathinones generally act as central nervous system stimulants by blocking the reuptake of monoamines in the brain, thereby increasing the concentration of neurotransmitters like dopamine, serotonin, and norepinephrine at the synapse (Coppola & Mondola, 2012b). The specific mechanism of action varies between compounds, and is generally produced either by blocking uptake (as with cocaine), or by acting as a transporter substrate and evoking the non-exocytotic release of transmitters from the pre-synaptic neuron via a process of reverse transport (as with amphetamine) (Baumann et al., 2012; Baumann et al., 2013; Cozzi, Sievert, Shulgin, Jacobill, & Ruoho, 1999; Eshleman et al., 2013; Nagai, Nonaka, & Kamimura, 2007; Schifano et al., 2011; Simmler et al., 2013). They produce the same CNS arousal as cocaine, amphetamines, and MDMA, though as their molecules are less lipophilic they are less able to cross the blood-brain barrier (although MDPV in particular is more able to cross the blood-brain barrier than other synthetic cathinones) (Coppola & Mondola, 2012a; Winder et al., 2013). The effects of bath salts appear to be dose-dependant, with MDPV being described as having stimulant effects similar to methylphenidate at low doses, and cocaine or methamphetamines at high doses

(Coppola & Mondola, 2012b; Zourob, 2011). The presence of a ketone on the molecule's structure reduces its central nervous system penetration and potency, and as a result users must consume a greater quantity of the compound (than amphetamine, for example) to achieve a similar effect. With greater quantities however, come more pronounced adverse effects and a greater abuse potential (Hill & Thomas, 2011; Winder et al., 2013).

1.3.4 Toxicology. Unfortunately, much of the evidence for the toxicological effects of bath salts has to date relied largely on self-report data and often cannot explicitly rule out other confounding variables (e.g., poly-drug use, incorrect drug identification, co-morbid medical or psychological conditions) (ACMD, 2010; Coppola & Mondola, 2012b; Dargan et al., 2011). The effects of acute toxicity appear to depend on the dose and frequency of use, and can include symptoms such as: psychomotor agitation, motor automatisms, parkinsonism, tremors, tachycardia, chest pains, hypertension, hyperthermia, mydriasis, dizziness, delusions, paranoid psychosis, depression, panic attacks, discolouration of the skin, long-term changes in cognition and emotional stability, rhabdomyolysis, abdominal pain, vomiting, kidney damage, hyponatremia, headache, cerebral edema, seizures, suicidal ideation, excited delirium, and severe insomnia (Baumann, 2014; Borek & Holstege, 2012; Coppola & Mondola, 2012b; Dargan et al., 2011). In hospitals acute toxicity is typically treated with benzodiazepines (to control agitation and seizures) and antipsychotics (to control severe agitation and psychotic symptoms). Hyperthermia is treated with aggressive cooling, and hyponatremia is treated with hypertonic saline and water restriction (Coppola & Mondola, 2012b; McClean et al., 2012; Spiller, Ryan, Weston, & Jansen, 2011).

There have been few studies to date looking into the potential long-term toxicity of bath salts (ACMD, 2010). One study by den Hollander et al. (2013) looked at the possible long-term behavioral effects of mephedrone on mice. They found that working memory performance on a T-maze spontaneous alternation task was reduced following a 4-day

'binge-like' regimen of mephedrone. This suggests that long-term sequelae of repeated bath salt use may indeed be a concern, though more research in this area is needed.

1.3.5 Fatalities. There have been a number of high-profile deaths reported in the media that have been 'linked' to bath salts, though in several of these cases the deaths have later been shown to be the result of other causes (or at least not solely due to bath salts, i.e., poly-drug use was involved) (Coppola & Mondola, 2012b; Dargan et al., 2011). Even so, there is a growing body of evidence reporting a number of deaths that are the direct result of synthetic cathinone use, usually attributed to cerebral edema (ACMD, 2010; Dargan et al., 2011; Maskell, De Paoli, Seneviratne, & Pounder, 2001).

1.4 Abuse Potential

Bath salt users have described strong cravings to re-use the drug and a number of researchers have found synthetic cathinones to have a similar abuse potential to amphetamines and MDMA (ACMD, 2010; Bonano, Glennon, De Felice, Banks, & Negus, 2014; Coppola & Mondola, 2012b; Creehan, Vandewater, & Taffe, 2015; Gatch et al., 2015; Hadlock et al., 2011; Hutsell et al., 2015; Schindler et al., 2015; Wood & Dargan, 2012). Mephedrone has been shown to have strong reinforcing properties, and is readily self-administered by rats (Aarde et al., 2013; Hadlock et al., 2011; Motbey et al., 2013). In a survey of poly-drug users, users of mephedrone were as likely as users of MDMA to self-report three or more DSM-IV symptoms of substance dependence (Uosukainen, Tacke, & Winstock, 2015).

In an experimental study by Lisek et al. (2012) acute mephedrone administration increased ambulatory activity in rats, consistent with the expected effects of similar psychostimulants. They found that ambulation was inhibited with pre-treatment with a dopamine D_1 antagonist and enhanced with pre-treatment with a dopamine D_2 antagonist. Following chronic treatment with mephedrone, the rats were then re-administered the drug

following 10 days of abstinence and they displayed sensitization to the psychomotor stimulant effects. Lisek et al. (2012) also performed conditioned place preference tests and found that mephedrone conditioning resulted in a preference shift for both rats and mice. They conclude from their study that their findings suggest an 'abuse liability' of bath salts similar to established drugs of abuse. In a similar study Gregg, Tallarida, Reitz, McCurdy, and Rawls (2013) also concluded that mephedrone produced sensitizing behavioural effects, though they found said effects to be weaker than those of established psychostimulant drugs.

Similar conclusions of abuse potential were drawn from a study by Robinson, Agoglia, Fish, Krouse, and Malanga (2012). In their experiment they compared mephedrone to cocaine in an intracranial self-stimulation (ICSS) paradigm in rats. After intraperitoneal administration of saline, mephedrone, or cocaine, rats were placed in an operant chamber with electrodes in their lateral hypothalamus. ICSS responding was monitored to gauge the effect each drug had on brain reward systems. They found that both mephedrone and cocaine dose-dependently decreased the half maximal responding threshold and the brain stimulation reward threshold of the rats. In addition, mephedrone also lowered the maximum responding rate, whereas cocaine did not. These findings suggest that mephedrone has similar effects to cocaine on reward systems in the brain; though interestingly the results from mephedrone treated rats only began 15 minutes after administration, indicating that it has a slower onset of action.

A further animal study compared methamphetamine, MDPV, MDMA, and mephedrone in their acute effects on locomotor wheel activity in rats (Huang et al., 2012). Results from this study found that MDPV was comparable to methamphetamine (they both produced a biphasic change in the pattern of wheel rotations after administration), whereas mephedrone was more comparable to MDMA (their observed reduction in wheel counts was monophasic and dose-dependent). These results are interesting because they suggest that the

effects of bath salts may be specific to the particular active ingredient used: MDPV may be primarily a psychomotor stimulant like methamphetamine, and mephedrone may be more like an entactogen, such as MDMA (ecstasy).

There is evidence to suggest that frequent use of high doses of bath salts can cause tolerance, dependence, craving, and withdrawal symptoms following cessation (Coppola & Mondola, 2012b). The most common withdrawal symptoms for mephedrone include fatigue, insomnia, nasal congestion, and impaired concentration; though they can also include depression, anxiety, increased appetite, irritability, unusual sweat odour, and urge/craving for use (Marsden et al., 2011; McClean et al., 2012).

Dargan et al. (2011) suggest that physical dependence on mephedrone is "unlikely" given its particular pharmacology. Instead, they suggest that any dependence-like syndrome and withdrawal symptoms are psychologically based (though they may require medical treatment). This view may also be supported by an experiment conducted by Angoa-Perez et al. (2012). In their study they found that mephedrone increased locomotor stimulation and hyperthermia in rodents, similar to amphetamines, though surprisingly it did not cause neurotoxicity to the dopamine nerve endings in the striatum (as methamphetamine does). This may suggest that mephedrone, while producing many of the same behavioural and physiological effects as amphetamine, does not pose the same physiological risk from long-term repeated use.

Given the frequency of poly-drug abuse amongst users, Berquist, Peet, and Baker (2015) tested mephedrone's ability to increase sensitisation to other drugs (namely, amphetamine). They found that rats treated with both mephedrone and amphetamine had significantly higher behavioural sensitisation indices compared to those treated with either mephedrone or amphetamine alone, following re-exposure to amphetamine after a 10 day

'wash out' period. This finding suggests that mephedrone use may enhance sensitivity to the behavioural effects of amphetamine.

1.5 Conclusion

Although most countries now have legislation banning the use of synthetic cathinones or 'bath salts', their sale and distribution is still commonplace. These drugs are relatively cheap and easy to acquire and are believed by many users to be safer (if not legal) alternatives to 'traditional' illicit drugs (like methamphetamine and ecstasy). Comparatively little is known about the effects and methods of action of bath salts, as research in this area has only recently begun to contribute to our knowledge base. What we know already suggests that while these drugs may be analogous to known psychoactive substances they are also different enough to defy some obvious expectations. That these drugs can be so easily obtained and used is of great concern, as their full impact both on the user and those around them is not yet known. Already bath salts have been implicated in a number of horrific high-profile crimes such as the case of a homeless Miami man caught allegedly attacking another man and eating his face (ABC News, 2012).

The suggestion that current knowledge makes is that bath salts likely have similar abuse potential to known drugs of abuse. There is little empirical evidence of this yet however, with most of the literature currently comprised of self-report data, emergency room reports, and poison control center call logs. The literature is filled with anecdotal evidence and case studies and it is critical that this evidence is tested in controlled experiments. It is important that we better understand what these drugs do, how they work, and what potential problems users might face (such as dependence and addiction). It is similarly crucial that public knowledge of these drugs is increased, because several commonly held beliefs about the purported safety of these designer drugs might be gravely misleading.

1.6 The Current Study

The current study was designed to contribute to the emerging, collective knowledge about bath salts, specifically the drug mephedrone. The scientific literature examining the effects of this drug is currently sparse relative to that of many more established illicit drugs, and this potentially has an impact on the ability of legislators and health professionals to combat and respond to what is a disturbing trend. Much of the current literature has a focus primarily on the neuro-pharmacological and toxicological effects, with little research into the behavioural sequelae of mephedrone use. Therefore, the current researcher chose to focus this study on the behavioural pharmacology of mephedrone over the course of three experiments. Using established animal models; Experiment 1 looks at the effects of mephedrone on anxiety-like behaviour in male and female hooded rats, including investigating the dose-response relationship. Experiment 2 assesses the effect of mephedrone on stereotypy; the persistent, repetitive behaviours observed in Experiment 1. And Experiment 3 looks at the potential long-term behavioural teratological effects of adolescent mephedrone use, and to what extent these effects can be mitigated by environmental enrichment. It is hoped that the combined findings of these three experiments will contribute a greater understanding of the behavioural effects of mephedrone, and any potential implications of these findings will be discussed.

2.0 Experiment 1 – Dose-Response Analysis

2.1 Anxiety - Overview

Anxiety is a commonly reported side effect of stimulant abuse and is well represented in the current toxicology literature as an effect of using mephedrone (e.g., ACMD, 2010; Dargan et al., 2011; Ross et al., 2011). In humans, anxiety is considered a psychological condition characterised by tension, expectation of an impending threat or disaster, and continuous vigilance for danger (Carlson, 2010). Exposure to anxiety-inducing stimuli causes activation of the sympathetic division of the autonomic nervous system, commonly referred to as the 'fight or flight' response (Cannon, 1963). In this state the body draws on stored energy reserves, releases epinephrine, and increases blood flow to the skeletal muscles in an effort to prepare itself for the physical demands of fighting or running away. 'Non-critical' systems (systems not contextually critical for assisting in an acute danger situation) like digestion are decreased to devote all possible resources to dealing with the current 'threat'. Once the danger has subsided, the parasympathetic nervous system (sometimes called the 'rest and digest' system) decreases heart rate and increases digestion, returning the body to its normal state of homeostasis (Carlson, 2010). The physical symptoms associated with anxiety can include shortness of breath, excessive sweating, irregular heartbeat, dizziness, fainting, and feelings of unreality. Psychological symptoms can include excessive worry, restlessness, irritability, poor concentration, and difficulty sleeping (Carlson, 2010; MHFNZ, 2016). Behaviourally, anxiety is associated with withdrawal and avoidance behaviours as people often try to alleviate their discomfort by removing themselves from the anxiety-inducing situation entirely (Friedman & Silver, 2006).

2.2 Animal Models of Anxiety

It is commonly held that anxiety in rodents is a comparable experience to that in humans (Ohl, 2003; Rowse, 2010). In response to a perceived threat a number of

physiological changes are triggered in the body, which in turn usually result in behaviour or behaviours intended to either remove the threat or remove oneself from the threat. These habits are observable in rats, which predictably exhibit anxiety-like behaviours, in response to threatening stimuli (e.g., an aversion to high, open spaces) (Palanza, 2001).

In behavioural testing, animal models of anxiety are typically comprised of either conditioned models or unconditioned models. Conditioned models involve training or conditioning the animals to respond to changes in their environment such as electric shocks, loud noises, or food or water deprivation (Rowse, 2010). These models are relatively time consuming, resource-intensive, and more invasive as the animals must be conditioned prior to testing, often over a period of days or weeks. Unconditioned models do not require any special training or manipulation to the animals; instead, they are observed more organically in situations that naturally induce an anxiety-like response (e.g., heights, bright lights, and open spaces) (Palanza, 2001). Many of these models exploit the natural conflict animals like rats have between their innate predisposition to explore novel environments, and their aversion to anxiety-producing situations. Common examples of behavioural tests for anxiety in rats include:

2.2.1 Open Field Test. Originally constructed as a large circular apparatus the 'open field' is now more commonly represented as a rectangular or square, lid-less box with a grid design on the floor. When placed in the apparatus the rat faces a conflict between their natural tendency to explore a novel environment, and their aversion to being 'vulnerable' in a wide, open space. As a result it is possible to both observe the rat's locomotor activity over time, and to infer its degree of anxiety based on its relative movements either in the inner grid squares (suggesting a less-anxious rat, content to explore the open areas) or the outer grid squares (suggesting a more anxious rat, keeping to the relative 'shelter' of the apparatus' walls) (Prut & Belsung, 2003).

2.2.2 Light/Dark Box Test. Constructed as a dual-compartment, enclosed box, this apparatus contains a 'light side' (a compartment painted white, with bright light entering) and a 'dark side' (a second compartment painted black, and darkened from any light source). When placed into the apparatus with free access to either side, the rat is faced with a conflict between its natural tendency to explore a novel environment, and its aversion to bright lights. The rat's degree of anxiety can then be inferred by the number of entries it makes into, and relative time spent in the 'aversive' light side (Bourin & Hascoet, 2003).

2.2.3 Elevated Plus Maze Test. Constructed as a plus shaped wooden maze and raised off the ground, the elevated plus maze apparatus is comprised of two closed arms (arms with high sides so the rat cannot escape) and two open arms (arms with no sides, so the raised arms are suspended over open space). When placed in the apparatus the rat is faced with a conflict between its natural tendency to explore a novel environment, and its innate fear of open and elevated spaces. The rat's degree of anxiety can be inferred by the number of entries into, and relative time spent in each of the arms (closed arms suggest more anxiety, open arms suggest less) (Silva & Brandao, 2000).

2.3 Sex Differences

In humans there are a number of sex differences noted in the prevalence, aetiology, and presentation of most psychological disorders (APA, 2013). In terms of anxiety there is a well-documented sex disparity for the prevalence of anxiety disorders, with females experiencing these syndromes nearly twice as often as males (Kokras & Dalla, 2014; Nemeth, Harrell, Beck, & Neigh, 2013). In spite of this accepted phenomenological fact the overwhelming majority of animal models of anxiety (and other disorders) have used only male subjects. This potentially brings in to question not only the generalisability of the studies' findings, but also the validity of the animal models themselves for examining female subjects (Kokras & Dalla, 2014; Palanza, 2001). The relatively few studies that have

attempted to observe sex differences in anxiety have often yielded inconsistent results and been unable to draw firm conclusions about which sex was more anxious (Johnston & File, 1991; Rowse, 2010). Females are typically more active in behavioural tests, and this significant difference in locomotion between the sexes can potentially be a confounding variable when trying to infer anxiety-like behaviours (Kokras & Dalla, 2014).

2.4 Known Effects of Other Stimulants

While no behavioural studies currently exist examining the effects of mephedrone on anxiety in rats, there have been a number of studies looking at the effects of similar stimulants of abuse. Methamphetamine, MDMA, cocaine, methylone, and benzylpiperazine (BZP) have all been shown to produce an observable anxiogenic effect (i.e., increased levels of anxiety relative to a saline control) in similar behavioural tests (Aitchison & Hughes, 2006; Daniel & Hughes, 2016; den Hollander et al., 2013; Pometlova, Nohejlova-Deykun, & Slamberova, 2012; Quinteros-Munoz et al., 2010; Thompson, 2012; Yang, Gorman, Dunn, & Goeders, 1992).

2.4.1 Dose-response relationships. When trying to understand new drugs on the market it is important to investigate their effects not only globally, but also how those effects may change at different doses of the drug. Sometimes a drug can be beneficial at low doses, but dangerous or even lethal at high doses. The beneficial effects may increase as the dose increases, or they may plateau (or even reverse) after a particular dose strength. The relationship between the dose and the subsequent effect(s) is commonly known as the 'dose-response relationship' (Moroney, 2016). Previous research has shown the dose-response relationship for the effect of similar stimulant drugs (e.g., caffeine, methamphetamine, and methylphenidate) on locomotor activity to be an inverted 'U' shaped curve (initially an increase in activity as drug dose increases, then a decrease) (Clemens, Cornish, Hunt, & McGregor, 2006; Hughes & Greig, 1976; Quinteros-Munoz et al., 2010).

2.5 Experiment 1 Aims and Hypotheses

Experiment 1 sought to examine the effects of mephedrone on anxiety-like behaviours in male and female hooded rats. While the scientific literature on the behavioural effects of mephedrone is still quite sparse, much of our current knowledge suggests that it behaves analogously to known stimulants like methamphetamine. As these stimulants have been shown in previous animal studies to be anxiogenic, it was hypothesised that mephedrone would similarly increase anxiety-like behaviours in rats, relative to saline controls. Additionally, Experiment 1 sought to investigate the dose-response relationship of mephedrone on anxiety-like behaviours. Using an increasing range of doses (1mg/kg, 20mg/kg, 40mg/kg, and 60mg/kg) as well as a saline control group, it was hypothesised that mephedrone would produce an inverted 'U' shaped curve (an initial increase in observable anxiety-like behaviours, followed by a decrease) similar to previous results from tests with comparable stimulant drugs.

3.0 Method

3.1 Subjects

The subjects used in this study were 50 male and 50 female PVG/c hooded rats bred in the Animal Facility of the Department of Psychology at the University of Canterbury, Christchurch, New Zealand. Following weaning at PND30 (Postnatal day 30) the animals were housed in 525 x 330 x 230mm-high plastic cages in groups of three or four same-sexed animals. They were kept on a 12-hour light/dark cycle (lights on at 0800 hours) at an ambient temperature of 22 ± 2°C (with humidity of 48% ± 10%). All subjects had free access to standard laboratory food and drinking water at all times. The care and experimental treatment of subjects complied with Parts 5 (Code of Welfare) and 6 (Use of Animals in Research, Testing and Teaching) of the New Zealand Animal Welfare Act, 1999 and had been approved by the Animal Ethics Committee of the University of Canterbury (See Appendix A).

3.2 Mephedrone Treatment

The subjects were randomly assigned to one of five drug treatment conditions, with each condition containing 10 male and 10 female subjects. The conditions were: 1mg/kg, 20mg/kg, 40mg/kg, or 60mg/kg of mephedrone, and a saline control group. This dose range was selected to approximate the range of dosages commonly used in the existing literature (e.g., den Hollander et al., 2013; Lisek et al., 2012; Marusich, Grant, Blough, & Wiley, 2012). The mephedrone used in this study was synthesised on-site in the Department of Psychology at the University of Canterbury. The resulting mephedrone 98% salt was dissolved daily in isotonic saline to create solutions of the various dosages. These doses were then administered to the subjects via intraperitoneal (I.P.) injections in a volume of 1ml/kg.

At approximately PND100 (adulthood) subjects received treatment once per day, for four non-consecutive days. The subjects were removed from their home cage, injected with the appropriate solution (for their experimental condition), and then placed in their own

separate, covered ‘holding cage’. Twenty minutes post-injection (when the drug, if administered, had taken effect) the subject was removed from their ‘holding cage’ and placed in the appropriate testing apparatus (see ‘3.3 Behavioural Testing’ section below). Following testing, subjects were returned to their home cages except in cases of continuing acute drug intoxication (e.g., excessive hyperactivity and hypersensitivity). In these cases they were instead returned to their own ‘holding cage’ for up to an hour (to allow for the acute effects of the drug to diminish) before then being returned to their home cage.

3.3 Behavioural Testing

Behavioural testing occurred 20 minutes following drug treatment (one test per day, with each testing day separated by a ‘rest’ day). All subjects were tested using four behavioural testing paradigms: an open field (OF), an elevated plus maze (EPM), a light/dark box (LD), and a novel object recognition (NOR) test. For each subject, tests were completed over seven days, with only one test being performed each day, and each testing day followed by a ‘rest’ day. The sequence in which the subjects performed the four different tests was counterbalanced based on their cage allocation. All tests were performed in one of two experimental rooms under low-light conditions.

Immediately following each individual subject’s test, the testing apparatus was thoroughly cleaned with a 20% Powerquat blue solution to attenuate confounding odour cues from previous trials. Further to this, on the days when both males and females were tested in the same apparatus, males were always tested first. This was because it has been suggested that males may be particularly sensitive to odour cues left behind by females even after thorough cleaning (Hughes, 2007).

All tests were observed via a small CCTV camera suspended over the apparatus. This allowed the experimenter to view the subjects from a television monitor some distance away

in the room (i.e., so they weren't standing over the apparatus) and reduced the likelihood of their presence affecting the subjects' behaviour.

3.3.1 Open Field – Apparatus. The open field consisted of a 600x600mm square wooden arena 250mm high. The interior walls and floor of the apparatus were painted black, except for white intersecting lines dividing the floor into 16 equal squares (creating a 4x4 grid pattern). Each square in the grid was numbered 1 to 16.

3.3.2 Open field – Procedure. At the beginning of the procedure the rat was placed into the center of the open field apparatus and 6 seconds later recording began. A behavioural sampling procedure was used whereby every 3 seconds (signalled by a small tone generator produced by the Smartphone app 'Encore', and delivered through an earpiece) the experimenter recorded two pieces of data on an OF record sheet (see Appendix B): 1) the subjects' location in the apparatus (as measured by which grid square the majority of the animal's body was occupying), and 2) what behaviour the subject was engaging in; either walking (W), rearing (R), freezing (F), or grooming (G). This continued for 5 minutes (100 observations).

High frequencies of walking, rearing, and center occupancy (frequency of the subject noted in one of the four center squares) are regarded as indices of low anxiety, whereas high frequencies of freezing, grooming, and corner occupancy (frequency of the subject noted in one of the four corners) are thought to indicate high anxiety (Prut & Belsung, 2003). Ambulation (distance travelled, a measure of activity) was later estimated by counting the number of transitions between 3-second observations (i.e., the number of times the subject was recorded as being in a different grid square to where it had been in the preceding interval).

At the end of the trial, the number of faecal boluses left in the apparatus ('defecation') was counted and recorded as a measure of emotionality (more boluses represent greater emotionality) (Palanza, 2001).

3.3.3 Elevated plus maze – Apparatus. The elevated plus maze stood 1 meter off the ground and consisted of four 500mm-long x 100-wide arms extending at 90° to each other from a central platform (150mm x 150mm). Two opposing arms had 245mm-high wooden walls that were painted black (the 'closed' arms) and the other two arms had walls instead constructed of clear Perspex (the 'open' arms). While typically an EPM has a distinct lack of walls on its 'open' arms (to create an aversive situation for the subjects to experience or avoid), the transparent arms on this apparatus prevent a startled rat from leaping off the 'open' arms and do not appear to reduce its aversiveness (Martinez, Cardenas, Lamprea, & Morato, 2002).

3.3.4 Elevated plus maze – Procedure. At the beginning of the test the subject was placed in the center platform of the maze, facing one of the open arms. Recording began 3 seconds later in a similar manner to the OF testing described above. Using an EPM record sheet (see Appendix C) the experimenter recorded the number of entries (all four feet) into each arm, and every 3 seconds noted whether the subject was occupying one of the open arms (O), one of the closed arms (C), or the center platform (X). It was then possible to calculate the subject's percentage of entries into and percentage of time spent in the open arms. These measures are regarded as evidence of the subject's willingness to enter the anxiety-invoking open areas of the EPM (Pellow, Chopin, File, & Briley, 1985). The number of entries into the closed arms was also noted as this represents a measure of general activity that is not confounded by anxiety (Cruz, Frei, & Graeff, 1994).

3.3.5 Light/dark box – Apparatus. The light/dark box consisted of a 600 x 200 x 250mm-high painted wooden box divided equally into two compartments. The left

compartment (light side) was painted white and had a clear Perspex lid to allow light to enter. The right compartment (dark side) was painted black and had a hinged lid to create total darkness when closed. There was a 100 x 100mm opening in the bottom of the wall dividing the two sides that could be opened or closed by means of a vertical guillotine slide.

3.3.6 Light/dark box – Procedure. At the beginning of the trial the subject was placed inside the dark compartment with both the lid and slider closed. After 15 seconds the slider was opened, allowing the subject free access to both compartments. Using the LD box record sheet (see Appendix D) the experimenter recorded the latency (in seconds) for the subject to make their first full emergence (all four feet) into the light compartment, and the frequency of partial emergences (at least one foot) before their first full emergence. Beginning from the time the slider was opened, the experimenter also recorded which compartment (L or D) the subject was occupying every 3 seconds for 5 minutes (100 observations).

Higher frequencies of entering the light side, greater percentage of time spent in the light side, and shorter first emergence latencies are all considered indices of lower anxiety (Bourin & Hascoet, 2003). The frequency of partial emergences (before the first full emergence) has been suggested to represent a degree of risk assessment in adult rats (Arrant, Schramm-Sapyta, & Kuhn, 2013).

3.3.7 Novel object recognition – Apparatus. The NOR test was conducted in the same apparatus as the OF. The ‘familiar’ objects used were two weighted 300ml drink cans. The cans were reflective gold in colour, 115mm-high and 60mm in diameter. The ‘novel’ object was a cream-coloured stop clock, 115mm x 115mm x 45mm-deep.

3.3.8 Novel object recognition – Procedure. The NOR test comprised two acquisition trials and one retention trial. The subject received drug treatment only before the retention trial. The first acquisition trial was designed solely to habituate the subject to the

OF apparatus. As such, the subjects' 5 minute exposures to the apparatus during their OF tests (which were always scheduled to occur before their NOR tests) were considered to account for their first acquisition trial for the NOR test. The second acquisition trial, designed to habituate the subjects to the 'familiar' objects, occurred two days later. For this trial the two cans were placed in opposite corners of the OF and the subject was entered into the apparatus. For 10 minutes the subject was free to move about the apparatus and investigate the objects. The subject was then removed from the OF and treated with I.P. mephedrone or saline (see '3.2 Mephedrone Treatment'). Twenty minutes following their treatment the subjects performed the retention trial. One of the two cans ('familiar' objects) was removed from the OF and replaced with the stop clock ('novel' object). The corners containing the novel and familiar objects were alternated between subjects to account for any positional or lighting preferences in the room. The subject was then placed into the centre of the OF and for 5 minutes, every 3 seconds the experimenter recorded on the NOR record sheet (see Appendix E) if the subject was in physical contact with, or proximity to the novel or familiar objects. 'Proximity' was classified as oriented towards and within 2cm of one of the objects. From this data a NOR discrimination index was calculated providing a single figure between -1 and 1. An index score of greater than zero indicates a preference for the novel object relative to the familiar object, whereas an index score of less than zero represents the opposite (Hughes, Hancock, & Thompson, 2015; Sutcliffe, Marshall, & Neill, 2007). The NOR discrimination index was calculated as follows:

$$\text{NOR discrimination index} = \frac{\text{novel object exploration} - \text{familiar object exploration}}{\text{total exploration}}$$

3.4 Novel object recognition – Test of preference

A small, independent experiment was performed with 20 adult PVG/c hooded rats (10 male and 10 female) to determine if there was an inherent preference for one object over the other (i.e., a can or a stop clock), irrespective of their novelty values. Similar to the procedure described above, one of each of the objects was placed into opposite corners of the open field and each subject was allowed five minutes to freely move about the apparatus. Exploration of each object was recorded in the same manner as in the NOR retention trial, and the mean±SEM total exploration of the can and the clock were found to be 10.25±1.33 and 9.15±1.25 respectively. This difference was not statistically significant [$F(1,36) = 0.46$, $p>0.1$], indicating that the subjects did not prefer either the can or the clock more than the other when both were equally novel. Female subjects were found to explore both objects more than males [females=12.55±1.25, males=6.85±0.97, $F(1,36) = 12.40$, $p<0.01$].

3.5 Non-Behavioural Measures

In addition to the behavioural measures each subject's weight and temperature were also recorded. Weight, in grams, for each subject was measured using standard laboratory scales on each of the four testing days (necessary to calculate the appropriate volume of solution to inject). The weight on the first day was subtracted from the weight on the last day to give a figure representing the change in weight for that subject over the seven days of testing. It was then possible to compare weight gain (or loss) over this time between the different experimental groups.

The subjects' temperature was also recorded, using a Braun IRT 4020 ThermoScan aural thermometer. Temperature recordings were taken on each testing day for all subjects. The first recording was taken at time of drug (or saline) administration, and the second was taken immediately following behavioural testing (i.e., 25 minutes following drug administration). The first temperature was subtracted from the second to give a figure

representing the change in temperature, and this ‘difference score’ was averaged for each subject over the four testing days. The final figure then was an average temperature change (in degrees Celsius) for each subject, and allowed for comparisons to be made between different experimental groups.

4.0 Results

Unless otherwise stated, all measures were statistically analysed using separate 5 (drug) × 2 (sex) ANOVAs, and Fisher PLSD post hoc comparisons ($p<0.05$). Where appropriate, ANCOVAs were also utilised in order to test the significance of a given result after accounting for a potentially confounding co-variable. In some specific cases, trend analyses were performed when visual inspection revealed potential patterns in the data. The experimental design, with 5 increasing doses of mephedrone, meant that the use of these tests in this manner was reasonable given the exploratory nature of the experiment (Cohen, 2008). All analyses were performed using Statistica 12 software by StatSoft©.

A number of subjects were excluded from the final analyses for various reasons: subject 95 (female, 60mg/kg) was excluded from the open field results due to 'seizure-like' activity during the testing. Subject 41 (male, 60mg/kg) was excluded from the elevated plus maze results due to an error in drug administration. Subject 26 (male, 20mg/kg) was excluded from the novel object recognition test results due to missing the required habituation trial. Subject 91 (female, 60mg/kg) experienced an adverse reaction to the drug treatment and was euthanized before participating in the novel object recognition test, and so did not contribute to the data pool for that particular test.

4.1 Open Field Results

The effects of increasing mephedrone doses on the behavioural measures examined in the open field are displayed in Figure 1. As can be seen: rearing and grooming behaviours, as well as defecation, decreased with higher doses of mephedrone; freezing behaviours and corner occupancy increased with higher doses; and ambulation, walking, and center occupancy all initially increased before peaking at 20mg/kg and then decreasing with higher doses.

It was posited that the center and corner occupancy measures might potentially be confounded by hyperactivity (e.g., a subject running quickly around the edges of the open field has more opportunity to be recorded as having occupied a corner square on any given observation). To assess this, these measures were also analysed used ANCOVA, with ambulation as a co-variable. The effects of mephedrone on both center and corner occupancy remained significant [$F(4,92) = 3.52$, $p < 0.05$; and $F(4,92) = 10.16$, $p < 0.001$ respectively].

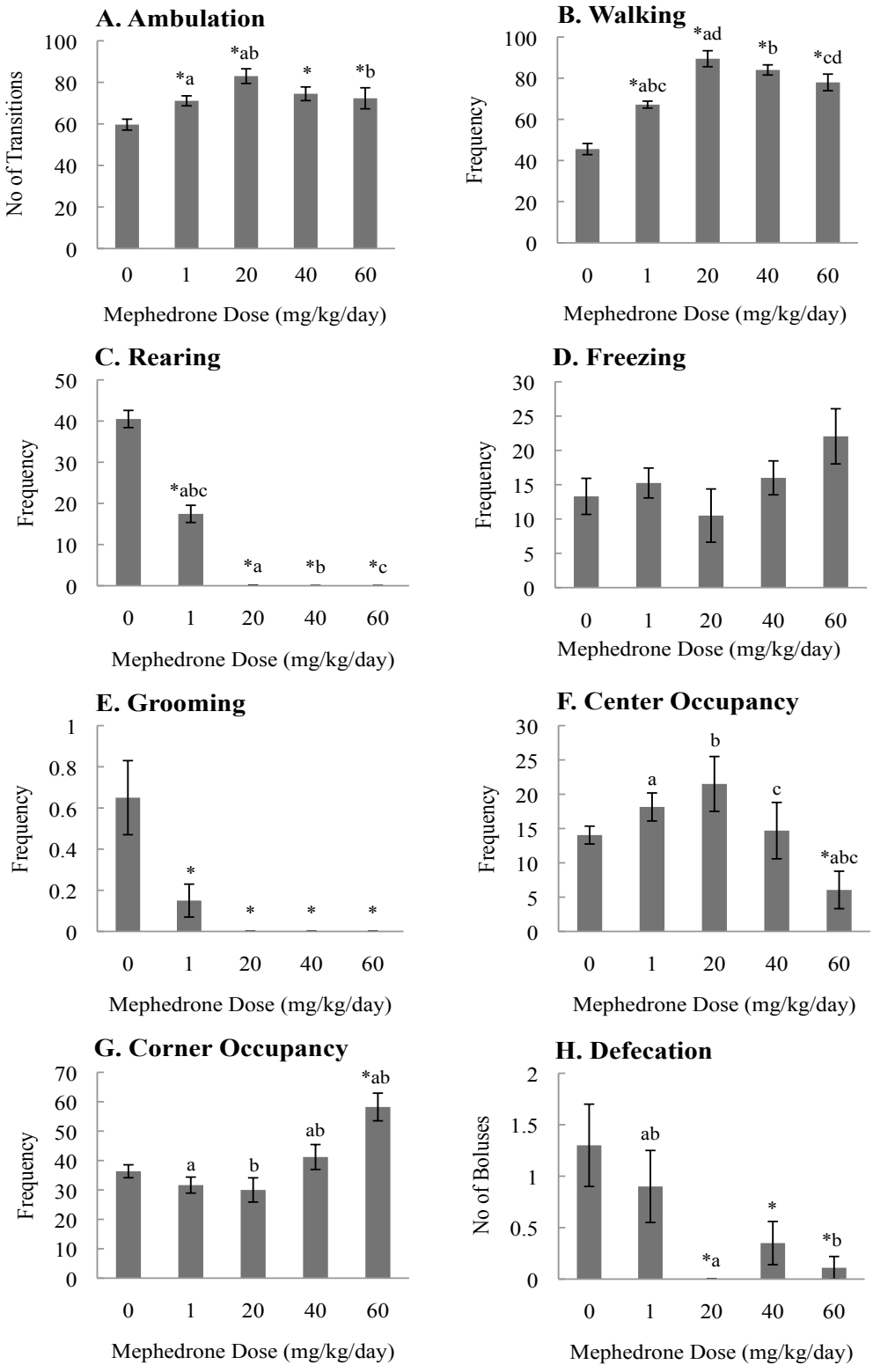

Figure 1. Mean ± S.E.M. frequencies for (A) ambulation, (B) walking, (C), rearing, (D) freezing, (E) grooming, (F) center occupancy, (G), corner occupancy, and (H) defecation recorded in the open field following treatment with four doses of mephedrone. Data represents results for both male and female rats combined.

*Significantly different from control group ($p < 0.05$).

[a,b,c]Groups with superscripts in common are significantly different ($p < 0.05$).

Of the 8 behavioural measures examined in the open field, 3 were found to have significant drug × sex interactions. The results for ambulation [$F(4,89) = 2.52$, $p < 0.05$], center occupancy [$F(4,89) = 3.62$, $p < 0.01$], and corner occupancy, [$F(4,89) = 3.60$, $p < 0.01$] are displayed in Figure 2.

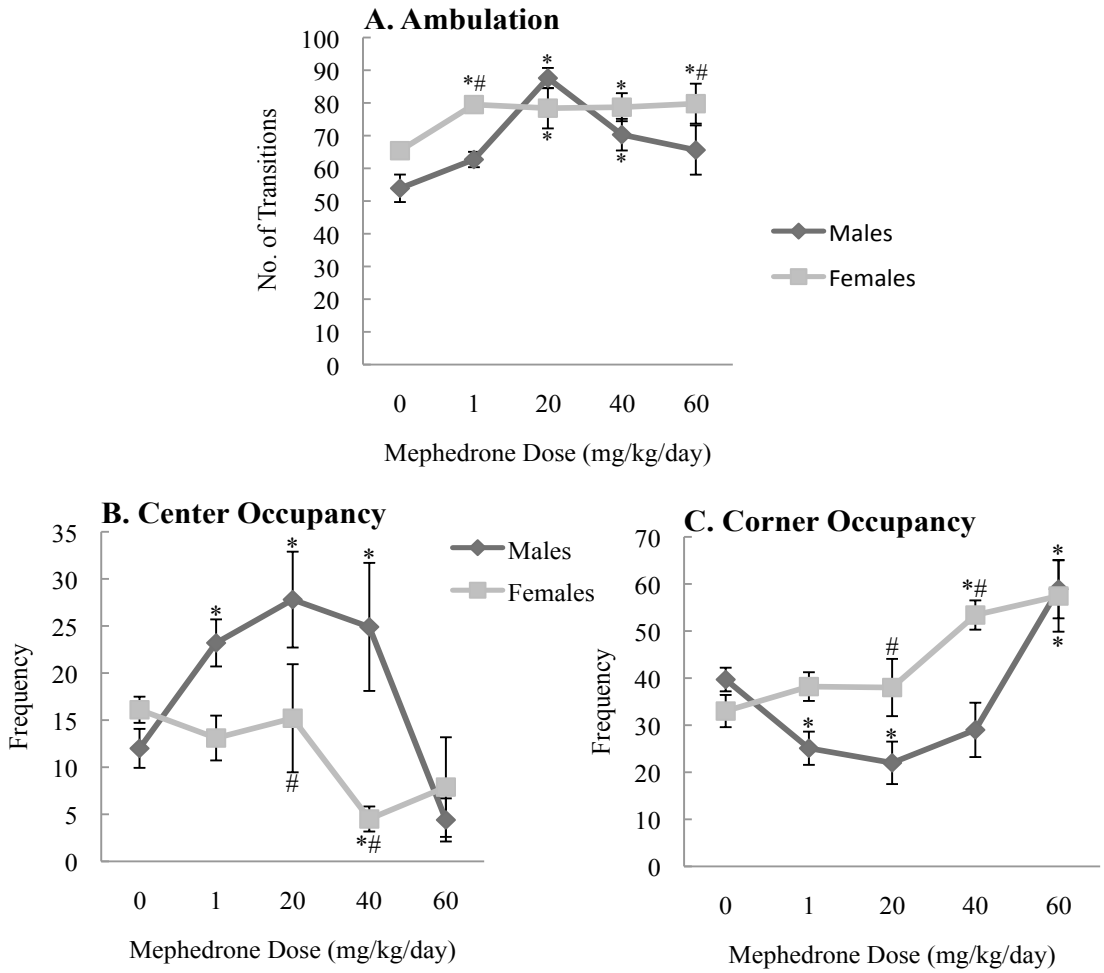

Figure 2. Mean ± S.E.M. frequencies for Sex × Drug interactions for (A) ambulation, (B) center occupancy, and (C), corner occupancy in the open field following treatment with four doses of mephedrone.
*Significantly different from control group for that sex ($p < 0.05$).
Signifies a significant difference between males and females for that dose ($p < 0.05$).

For ambulation and center occupancy, male subjects displayed first an increase, then a decrease in each behaviour as mephedrone dose increased (peaking at 20mg/kg), whereas females appeared to display a consistent increase in ambulation and decrease in center

occupancy. For corner occupancy, males displayed a decrease followed by an increase, whereas females displayed a consistent increase. Upon visual inspection of these findings it appeared that males and females responded in two distinct patterns on each measure. To test this, a number of trend analyses were performed. It was found that the pattern of responding for males followed significant quadratic trends for ambulation [$F(1,89) = 7.43$, $p<0.001$], center occupancy [$F(1,89) = 6.32$, $p<0.001$], and corner occupancy [$F(1,89) = 9.88$, $p<0.001$] measures. For females, a linear pattern of responding was significant for the corner occupancy measure [$F(1,84) = 4.86$, $p<0.01$], though not for ambulation or center occupancy [$F(1,84) = 1.79$, $p=0.14$; and $F(1,84) = 1.56$, $p=0.18$ respectively].

4.2 Light/Dark Box Results

Figure 3 shows the effects of increasing doses of mephedrone on subjects' behaviour in the light/dark box. As can be seen, mephedrone decreased the percent observations in the light side, as well as decreasing the number of entries into the light side up to a dose of 20mg/kg. For doses higher than 20mg/kg the direction of this effect was reversed, with observations and entries increasing. In contrast, the first emergence latency increased dramatically in doses up to 20mg/kg, before decreasing in doses above that point. 1mg/kg was the only dose that had a significant impact on the number of prior emergences into the light side.

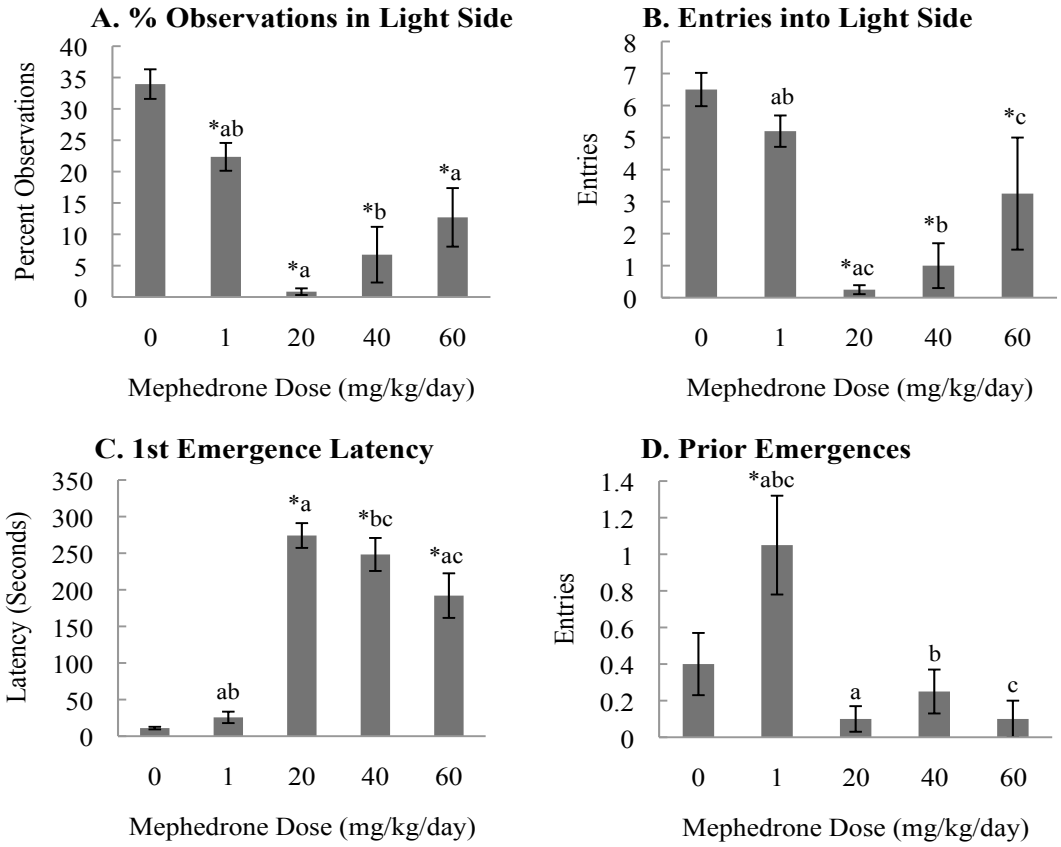

Figure 3. Mean ± S.E.M. results for (A) percent observations in the light side, (B) number of entries into the light side, (C), first emergence latency, and (D) number of prior, partial emergences into the light side recorded in the light/dark box following treatment with four doses of mephedrone. Data represents results for both male and female rats combined.
*Significantly different from control group ($p < 0.05$).
[a,b,c]Groups with superscripts in common are significantly different ($p < 0.05$).

Following visual inspection of the male and female data for these measures a number of potential patterns were explored using trend analysis. For entries into the light side male subjects showed a significant linear trend [$(F1,90) = 4.41$, $p<0.01$] with number of entries decreasing as mephedrone dose increased, whereas females showed a significant quadratic trend [$(F1,90) = 6.11$, $p<0.001$], displaying first a decrease, then an increased after 20mg/kg. The same patterns were also observed in the percent observations in the light side measure [Linear: $F(1,90) = 9.44$, $p<0.001$ and Quadratic: $(F1,90) = 9.91$, $p<0.001$, respectively].

4.3 Elevated Plus Maze Results

As can be seen in Figure 4, increasing doses of mephedrone had no significant effect on the percent observations in, or percent entries into the open arms of the elevated plus maze. The number of entries into the closed arms was significantly decreased at doses of 20mg/kg and higher. Factoring out entries of closed arms (an activity measure considered uncontaminated by anxiety) from percent entries of open arms did not alter the results enough to detect a significant effect of drug [$F(4,92) = 1.03$, $p=0.40$].

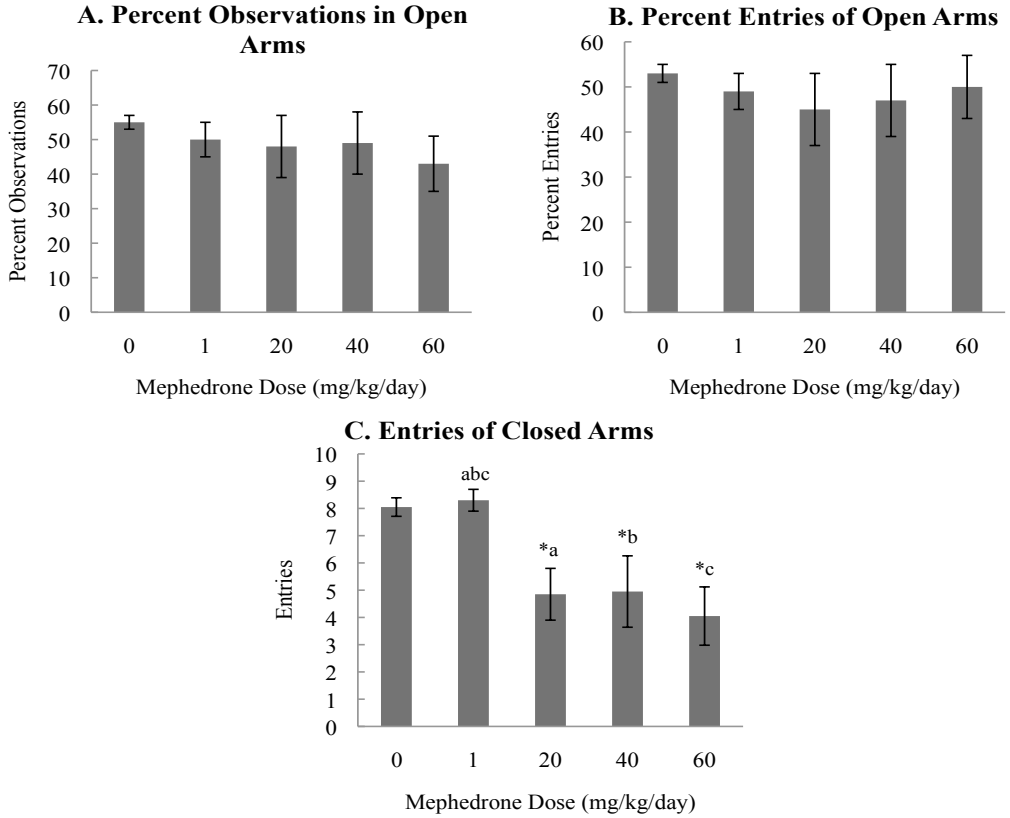

Figure 4. Mean ± S.E.M. results for (A) percent observations in the open arms, (B) percent entries into the open arms, and (C), number of entries into the closed arms recorded in the elevated plus maze following treatment with four doses of mephedrone. Data represents results for both male and female rats combined.
*Significantly different from control group ($p < 0.05$).
[a,b,c]Groups with superscripts in common are significantly different ($p < 0.05$).

A significant drug × sex interaction was found for the number of entries into the closed arms measure [$F(4,89) = 5.44$, $p < 0.001$]. As can be seen in Figure 5, the number of entries appears to be trending down as the mephedrone dose increases, except for the notable increases at 40mg/kg for males (but not for females), and at 60mg/kg for females (but not for males).

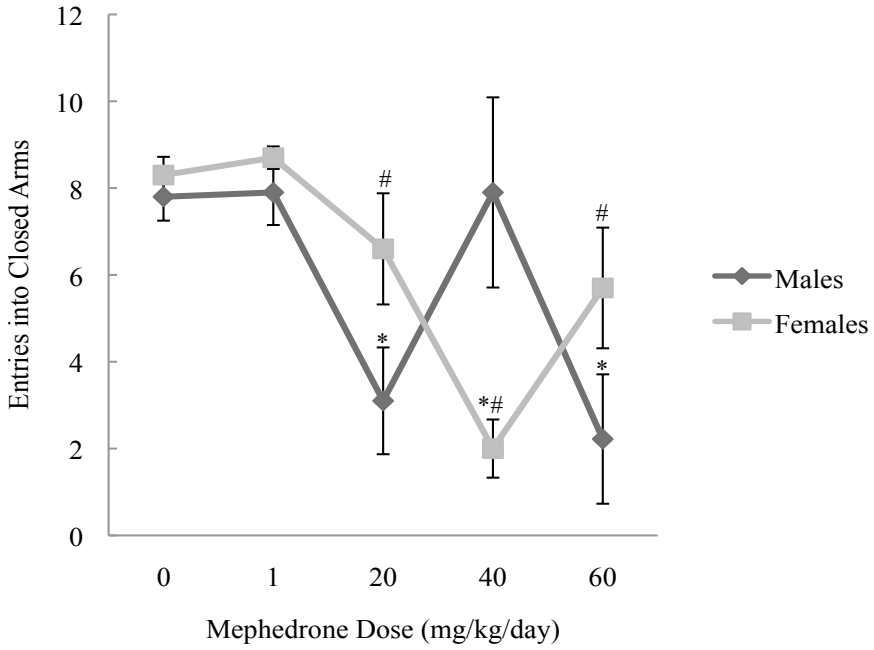

Figure 5. Mean ± S.E.M. frequencies for Sex × Drug interactions for total entries into the closed arms of the elevated plus maze.
*Significantly different from control group for that sex ($p < 0.05$).
Signifies a significant difference between males and females for that dose ($p < 0.05$).

4.4 Novel Object Recognition Results

The effects of mephedrone on the novel object recognition discrimination index (see 3.3.8 Novel Object Recognition - Procedure) are displayed in Figure 6. As can be seen, the discrimination index is greater than zero for each group, indicating that regardless of experimental condition there was a preference for exploring the novel object relative to the familiar one. Mephedrone dose failed to produce any significant overall effects [$F(4,85) = 1.59$, $p=0.18$], as did sex [$F(1,85) = 1.46$, $p=0.23$], though the sex × drug interaction was significant, [$F(4,85) = 4.72$, $p<0.01$] with males trending higher on the discrimination index at 60mg/kg, whereas females showed a decrease.

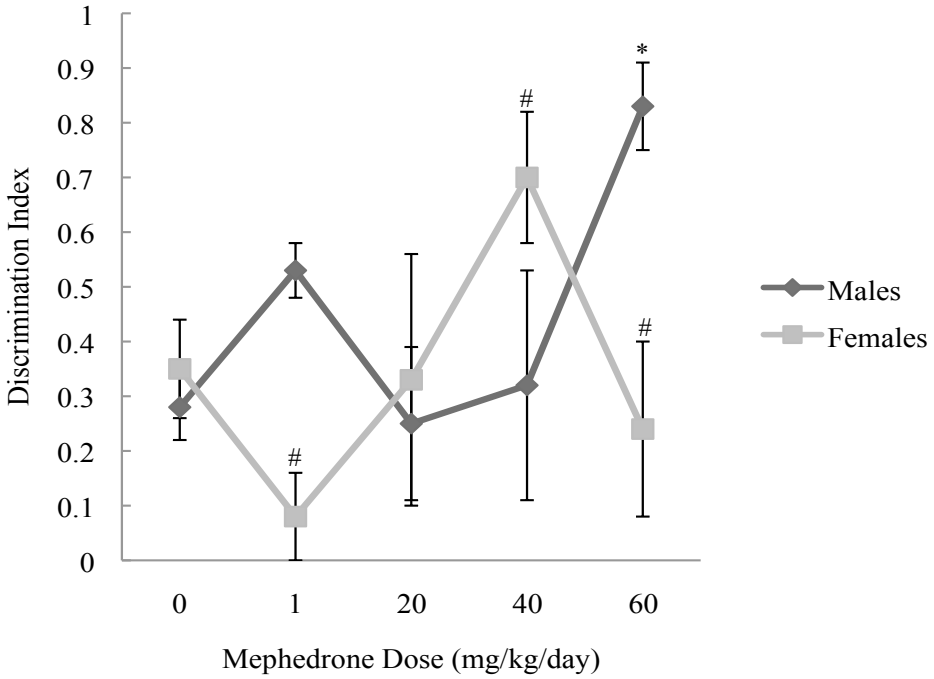

Figure 6. Mean ± S.E.M. results for male and female scores on the discrimination index in the novel object recognition test.
*Significantly different from control group for that sex ($p < 0.05$).
Signifies a significant difference between males and females for that dose ($p < 0.05$).

4.5 Sex Differences

For each measure recorded in the four behavioural tests overall sex differences were also examined, averaging scores across mephedrone (and saline) doses. The descriptive statistics and ANOVA results for males and females on each measure are summarised in Table 2. Female subjects travelled significantly further distances (ambulation) than males, and spent more time in the corner squares and less time in the center squares of the open field. They also made more entries into the light side of the light/dark box, and were observed spending more time in the light side compared to males. For most of the non-significant measures the frequencies of observed behaviours were generally higher for females than for males, such as walking and rearing in the open field, and entries into open and closed arms in the elevated plus maze.

Table 2
Means, S.E.Ms, and ANOVA results for main effects of Sex on all measures.

	Sex of Rats		
Measure	Male	Female	F(1,56)
Open Field			
Ambulation	68.02(±2.59)	76.29(±2.06)	8.21**
Walking	71.92(±2.86)	73.27(±3.05)	0.46
Rearing	10.78(±2.33)	12.67(±2.55)	1.87
Freezing	17.18(±1.79)	13.49(±2.18)	1.70
Grooming	0.12(±0.06)	0.20(±0.07)	0.96
Centre Occupancy	18.46(±2.20)	11.43(±1.70)	8.08**
Corner Occupancy	34.94(±2.78)	43.73(±2.51)	8.97**
Defecation	0.70(±0.19)	0.37(±0.16)	2.11
Light/Dark Box			
1st Emergence Latency (Seconds)	164.72 (±19.84)	135.76 (±19.44)	3.03
Entries into Light Side	2.24 (±0.38)	4.24 (±0.82)	6.52*
Observations in Light Side	10.92(±1.96)	19.72 (±3.01)	10.23**
Partial Entries into Light Side	0.38 (±0.11)	0.38 (±0.12)	0.00
Elevated Plus Maze			
% Entries in Open Arms	44% (±4%)	53% (±3%)	2.79
% Observations in Open Arms	45% (±5%)	54% (±4%)	2.03
Entries into Closed Arms	5.86 (±0.34)	6.26 (±0.52)	0.42
Novel Object Recognition			
Discrimination Index	0.44(±0.06)	0.33(±0.07)	1.46

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

4.6 Non-Behavioural Measures

The average difference in the subjects' weight between the first and last testing days (PND100 to PND107) is displayed for males and females separately in Figure 7. The results indicate that male subjects receiving doses of 20mg/kg or less of mephedrone gained between 6 and 10 grams over the testing cycle, whereas those receiving high doses of mephedrone gained significantly less weight (and in the case of the high dose group, lost weight). Female subjects appeared to gain between 0 and 4 grams, and there was no significant increase or decrease from control for any dose. This was a significant drug × sex interaction, $F(4,89) = 7.05$, $p<0.001$.

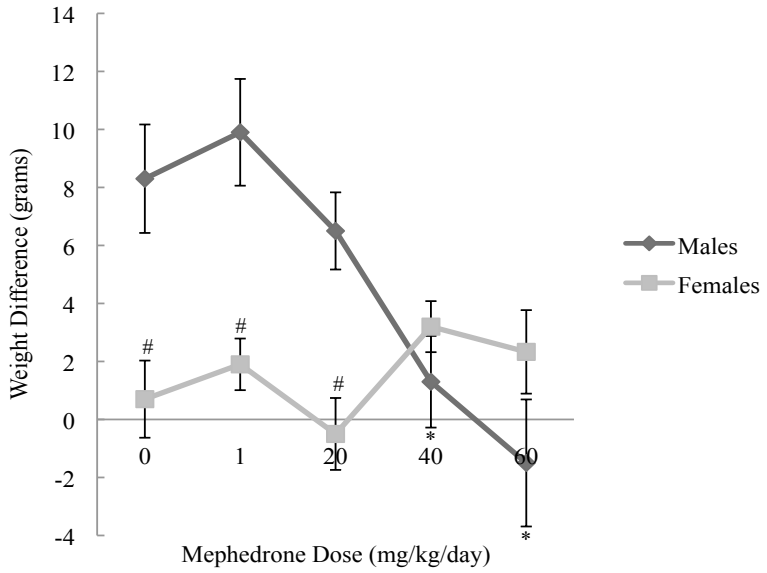

Figure 7. Mean ± S.E.M. results for weight difference (in grams) over the 7 days of testing for both male and female subjects in each experimental group.
*Significantly different from control group for that sex ($p < 0.05$).
Signifies a significant difference between males and females for that dose ($p < 0.05$).

Temperature data were recorded pre- and post-drug administration, and averaged across testing. Figure 8 displays the average temperature increase for male and female subjects 20 minutes after injection. As can be seen, both sexes experienced an increase in body temperature at all doses; however, female subjects showed an increasing difference as the mephedrone dose increased, whereas the increase for males appeared to peak at 20mg/kg.

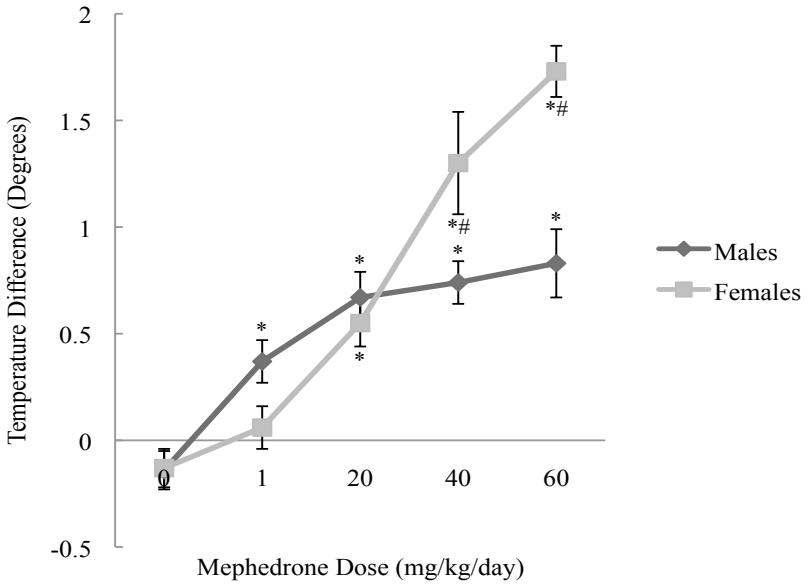

Figure 8. Mean ± S.E.M. results for average temperature difference (in degrees Celsius), 20 minutes following treatment with mephedrone for both male and female subjects.
*Significantly different from control group for that sex ($p < 0.05$).
[#] Signifies a significant difference between males and females for that dose ($p < 0.05$).

5.0 Discussion

Experiment 1 examined the anxiogenic properties of mephedrone over a range of doses. Both male and female subjects were administered 1, 20, 40, or 60 mg/kg of mephedrone (or saline) before being tested in an open field, light/dark box, elevated plus maze, and novel object recognition behavioural paradigm. The results of these tests supported Hypothesis 1 that mephedrone would be anxiogenic, producing more anxiety-related behaviours relative to the saline control group. Hypothesis 2 concerned the dose-response relationship between mephedrone and anxiety-like behaviours. It was predicted that this relationship would produce an inverted 'U' shaped curve (an initial increase in observable anxiety-like behaviours, followed by a decrease). The results of testing partially supported this hypothesis, though there were some interesting sex differences in the dose-response trends for several tests.

5.1 Open Field Findings

Mephedrone produced an overall increase in corner occupancy in the open field; as well as a decrease in rearing, grooming, and center occupancy. Taken together, these results are indicative of heightened anxiety, support Hypothesis 1, and are consistent with previous studies examining anxiogenic drugs (e.g., Aitchison & Hughes, 2006; Pometlova et al., 2012; Quinteros-Munoz et al., 2010; Thompson, 2012). While there was no statistically significant change in freezing behaviours, the data does appear to be trending upwards with high doses of mephedrone, which would similarly suggest increased anxiety.

The ambulation and walking measures also displayed significant increases following treatment with mephedrone. While higher scores on these two measures do not typically indicate increased anxiety, these results are consistent with the increase in locomotor activity generally observed following treatment with comparable psychomotor stimulant drugs (e.g.,

Gatch et al., 2015; Sahakian, Robbins, Morgan, & Iverson, 1975). This increased locomotor activity may also explain the lack of significant findings for the freezing behaviours measure.

The results for defecation, a measure thought to represent levels of emotionality (Anderson & Hughes, 2008), show a significant decrease following treatment with mephedrone, which runs contrary to the hypothesis and the other results. This unexpected finding however, can potentially be explained by the methodology of this particular experiment: because subjects were tested 20 minutes following drug administration (in order to allow for the drug to take full effect) it is entirely plausible that these subjects, made anxious by the oncoming drug effects, defecated in their temporary holding cages prior to being placed in the testing apparatus. Conversely, the control subjects, experiencing no anxiogenic effects of drug treatment and comfortable in their dark holding cages, would instead defecate only after being introduced to the anxiety-inducing testing apparatus. Given that the number of faecal boluses was counted only in the testing apparatus and not the holding cages, it may be that this measure is not valid with the present methodology.

When looking at the results across the dose range tested; ambulation, walking, and corner occupancy measures all show initial increases, peaking at 20mg/kg, before decreasing. The corner occupancy measure appears to display the opposite (a decrease, followed by an increase) though as lower levels of this measure represents more anxiety we can interpret this result to be the same as the others. Taken together these results would appear to support Hypothesis 2 (an inverted 'U' shaped dose-response curve), however, when the results for males and females are examined separately an interesting phenomenon can be observed. The predicted 'U' shaped curves for ambulation, center occupancy, and corner occupancy (later found to be significant quadratic trends) are found for male subjects only. Female subjects, conversely, appear to display a more linear dose-response relationship with higher doses of mephedrone producing greater levels of anxiety-like behaviours. That this finding is

replicated (albeit inversely) in both the center and corner occupancy measures adds validity to the detected effect, and suggests it is not simply an artefact of the data. The effect has not been found in previous studies, which suggests that either it is unique to mephedrone, or more likely, it is a consequence of the research literature's traditional use of male-only experimental designs (Hughes, 2007; Kokras & Dalla, 2014).

The inverted 'U' shaped curve found in previous research has been suggested to be a result of stereotypy behaviours contaminating the behavioural measures at higher doses (Clemens et al., 2006). This explanation is consistent with observations made by the experimenter in the current study, wherein subjects on higher doses of the drug were observed: tracing the outside walls of the apparatus ('thigmotaxis'), turning repeatedly in tight circles ('gyration'), and engaging in repetitive head swaying behaviours. If performed frequently enough, these behaviours could plausibly confound several of the behavioural measures recorded in the open field: for example, one 60mg/kg subject spent the entire 5 minutes of the test running clockwise around the exterior boundary of the apparatus. This animal would then be disproportionately likely to be recorded as having occupied a corner square on a given observation, even though doing so was largely an artefact of increased thigmotaxis rather than 'hiding' in the relative shelter of the corner. Center occupancy would be likewise affected, as an animal tracing the boundary of the apparatus would have no opportunity to be observed occupying the center squares.

These notes on stereotypy must be considered when interpreting the results of the experiment, though they do not necessarily mean that the initial findings are invalid. Increased anxiety may be strongly correlated with increased stereotypy, which would go some way to explaining the inverted 'U' shaped curve: anxiety increases beyond the peak, and accordingly so does stereotypy, potentially resulting in behaviours that erroneously represent decreased anxiety. Furthermore, if females displayed fewer stereotypic behaviours,

or at least overtly responded in a different way, this theory could explain the unexpected sex differences found in the dose-response relationships. It may be that higher doses of mephedrone produce greater anxiety for both male and female subjects, though after a certain point (in this case, 20mg/kg) the two sexes differ in their overtly observable behaviours (namely, stereotypy).

5.2 Light/Dark Box Findings

In the light/dark box, mephedrone decreased the percent observations in and number of entries into the light side of the apparatus, as well as increasing the first emergence latency. As with the open field findings, these results in the light/dark box suggest increased anxiety following treatment with mephedrone compared to saline control. These findings support Hypothesis 1, and are consistent with results from the drug literature (Bourin & Hascoet, 2003). The number of partial emergences made prior to the first full emergence has been suggested to be a measure of risk assessment behaviour. Interestingly, in this experiment only the 1mg/kg dose produced a significant increase in the number of partial emergences, with no differences seen at the higher doses. It is possible that at this lowest mephedrone dose the subjects, while not 'anxious' per se, could detect that 'something' was not normal and accordingly adopted a more cautious approach to investigating the (novel) light side of the apparatus. While the control subjects had little trouble entering the light side, the more anxious (higher dose) subjects appear to have taken a more 'all or nothing' approach to entering. It may be that at a low dose mephedrone induces a general or vague sense of discomfort that translates into increased risk assessment and cautionary behaviours, not unlike a mildly intoxicated driver being excessively careful because he understands he may be impaired.

The dose-response relationship seen in the light/dark box results is similar to that in the open field: the percent observations in the light side, and number of entries into the light

side measures both produced an inverted 'U' shape curve indicating an initial increase in anxiety-like behaviours followed by a decrease. These findings would appear to support Hypothesis 2, although when male and female results are viewed separately another sex difference emerges (again similar to the open field results). This time it is the females who display an inverted 'U' shaped, 'quadratic' trend for these two measures, whereas the males display a linear trend (higher doses translates to more anxiety). A surprising result, this finding may best be explained by the same theory put forward to explain the open field differences: that higher doses of mephedrone cause more stereotypy behaviours, and that these stereotypy behaviours are expressed differently for males and females. If stereotypy in males is expressed via relatively more non-ambulatory behaviours (such as head swaying) than females (who express stereotypy via more ambulatory behaviours such as thigmotaxis), then such stereotypy would result in different findings in the open field (where more locomotor activity generally increases scores, and increased scores on several measures represents more anxiety) than the light/dark box (where *decreased* locomotor scores indicate higher anxiety). For example, a male subject that sits still and engages in stereotypic head swaying for the entire 5 minutes of each test would likely score low on anxiety across the open field measures (little movement means less chance of being observed performing anxiety-like behaviours), whereas the same subject would score high on anxiety in the light/dark box (with few, if any, entries into the light side). Females, on the other hand, are already known to be more ambulatory than males (Archer, 1975; Johnston & File, 1991), and if their stereotypy behaviours reflect this then it follows they would appear to score lower on anxiety in the open field, and higher in the light/dark box compared to males at high doses.

5.3 Elevated Plus Maze Findings

Mephedrone did not significantly affect the percentage entries into the open arms, or percentage time spent in the open arms of the elevated plus maze. This is an unusual finding,

as previous studies conducted with similar drugs would lead us to expect mephedrone to decrease the entries into and time spent in the open arms respectively (Cruz et al., 1994; Martinez et al., 2002; Pellow et al., 1985; Silva & Brandao, 2000). The lack of significant findings here could be explained by one of the following explanations. (A): mephedrone does not affect subjects' preferences for either the open or closed arms in an elevated plus maze, and the current results accurately reflect this. This explanation seems unlikely given the results of the open field and light/dark box, which both suggest mephedrone produces anxiogenic effects, and more anxiety should translate to a preference for the closed arms in the elevated plus maze. (B): mephedrone does produce an effect on these measures, but the methodology of the current study is somehow flawed and so failed to detect it. This explanation also seems insufficient, as the current methodology used is an exact replication of that from several previous studies where significant effects were detected (Hughes, Hancock, Henwood, & Rapley, 2014; Hughes et al., 2015). (C): Another factor, unforeseen but unique to this study, has impacted on the ability of the test to detect an effect. This explanation seems plausible in light of the stereotypy observations already discussed for the open field and light/dark box. It may be that the elevated plus maze is more sensitive to the confounding effects of stereotypy than these other tests, and this is again consistent with observations made by the experimenter. Notably, many high-dose subjects, when placed in the apparatus would run to the end of the first arm they faced, and then pause, swaying their heads back and forth for the entirety of the test. If stereotypy is positively correlated with anxiety, and stereotypy also confounds observable anxiety measures in the elevated plus maze, then the lack of significant effects detected in the current study could strangely enough be plausible evidence of an anxiety response.

The number of entries into the closed arms of the elevated plus maze was included as a control measure to examine levels of locomotor activity. Doses of mephedrone 20mg/kg or

more, produced a significant reduction of entries into the closed arms, indicating that at these higher doses the subjects were less active (as measured by the test). This is not inconsistent with the above theory, as the increase in stereotypy behaviours would correlate with a decrease in 'detectable' locomotion (in this case, arm entries) if the animals are instead (A): not moving while they sway their heads back and forth; or (B): running in tight circles ('turning on the spot').

Taken together, the results from the elevated plus maze are somewhat inconclusive, though they could plausibly support Hypothesis 1 that mephedrone produces an anxiogenic effect, if stereotypy is taken into consideration. There is no evidence of a dose-response relationship to support Hypothesis 2.

5.4 Novel Object Recognition Findings

While not strictly a measure of anxiety, the novel object recognition task was added to examine the effects of mephedrone on recognition memory. The results showed that all subjects displayed a preference for the novel object relative to the familiar one, regardless of experimental condition, suggesting that mephedrone does not affect recognition memory. It should be noted however, that once again stereotypy might potentially have confounded some of the results, the experimenter noted that it was difficult at times to make a subjective determination as to whether an animal was 'actively exploring' an object (close to and oriented towards it) or simply 'head swaying' in its vicinity. With no quantitative way to factor out stereotypy effects, the results must be interpreted carefully, with this possibility in mind.

5.5 Sex Differences

In addition to those already discussed, there were a number of overall sex differences noted across the behavioural tests. Females displayed significantly more ambulation and corner occupancy in the open field, as well as less center occupancy. These three results are

indicative of females being generally more anxious than males, however; in the light/dark box females made more entries into the light side and were also observed more frequently in the light side, indicative of less anxiety than males. There were no significant sex differences found in the elevated plus maze or novel object recognition tests. These seemingly contradictory findings are consistent with previous research that has failed to conclusively find one sex to be more anxious than the other (Johnston & File, 1991). Rather than reflecting anxiety, specifically, the competing results from the open field and light/dark box (females are more anxious, and less anxious respectively compared to males) may be a product of locomotor activity. Females are well known for being more ambulatory than males (Archer, 1975), and the nature of the test measures is such that greater ambulation in the open field lends itself to more anxiety-like responses, whereas in the light/dark box the opposite is true (greater ambulation translates to less anxiety-like scores).

5.6 Non-Behavioural Measures

Weight gain over the seven days of testing, as well as average temperature increase post-injection were also recorded in this experiment. Mephedrone did not appear to affect the weight gain of female subjects, which put on between 0g and 3g consistently across the various doses. Male subjects however, were significantly affected by treatment with mephedrone, with 40mg/kg subjects only gaining between 1g and 2g over the week (compared to 8g or 9g for saline control subjects), and some 60mg/kg animals actually lost weight. It is unclear from the current study why mephedrone would produce this effect, though it could be the result of: loss of appetite, increased locomotor activity, increased metabolic rate, or a combination of these and other factors.

The aural temperature of the subjects was recorded at the time of injection, and again 25 minutes later immediately following behavioural testing. The difference in these temperatures, averaged across the four drug treatments, was calculated to assess the effect of

mephedrone on the subjects' temperature. The results showed that mephedrone significantly increased the temperature of the subjects at all doses, though the effect was different for male and females. Male subjects' temperatures were raised by over half a degree at 20mg/kg, though this increase levelled off and was similar for the higher doses as well. Females' temperatures showed a similar increase at 20mg/kg, though this increase was more linear, continuing to increase at higher doses until being over 1.5 degrees at 60mg/kg.

5.7 Adverse Effects

There were a number of adverse effects of mephedrone on some subjects recorded by the experimenter. These effects occurred almost entirely at doses in excess of 20mg/kg, and primarily in female subjects. Most notably, 3 high-dose female subjects experienced seizure-like activity during behavioural testing. The animals would suddenly freeze, as if startled, and then jerk violently for upwards of 10-20 seconds. Afterwards they appeared exhausted and panted motionlessly for a time. One of these animals was later euthanized when it failed to respond to the interventions of the animal technicians. The other two made full recoveries after rest and fluids. A general pathology report on the deceased animal did not return any remarkable findings, and it was posited by the animal technicians and the researcher that hyperthermia was likely the proximate cause.

A number of animals were found to have wet fur under their chins following behavioural testing (see Figure F1 in Appendix F). After a quick search of the literature this was identified as a condition called 'heat prostration'. Usually a result of rats being shipped in a high ambient temperature, the condition causes increased respiration and excessive salivation resulting in wet mouths, muzzles, and paws. The condition is thought to be a response by the rat of applying saliva to the fur in an attempt to cool down (Sharp & La Regina, 2010). The observable presence of this condition, as well as the temperature data

discussed earlier, is further evidence of the very real risk of hyperthermia when using high doses of mephedrone (particularly for females).

Four animals developed abscess-like wounds at the I.P. injection sites. While alarming and gruesome to observe (see figure F2 in Appendix F), the sores did not seem to cause the subjects undue discomfort (i.e., they did not respond when the sores were touched). The sores were monitored, and healed themselves over a period of 1-3 weeks. The subjects concerned were males and females, from either the 40mg/kg or 60mg/kg experimental groups. It is not clear why some animals developed the sores while the majority of others did not.

Other less extreme, though no less interesting effects were also noted at high doses of mephedrone use. Subjects treated with over 20mg/kg frequently exhibited agitation, increased locomotor activity, hypersensitivity to touch and to sound, and exaggerated startle reflex. The high dose rats were often difficult to handle, sometimes requiring the researcher to gently wrap them in a tea towel in order to pick them up. Many of these symptoms are consistent with the literature reporting the effects of mephedrone use in humans (see Table 1, page 6).

5.8 Limitations and Future Research

On examination and discussion of the results from Experiment 1, the primary methodological limitation is almost certainly the lack of any quantitative means of assessing (and therefore factoring out) stereotypy behaviours. While not necessarily invalidating the current findings, increased stereotypy is clearly a significant effect of mephedrone (particularly at high doses) and a large factor that was not controlled for in the current study. A number of the conclusions drawn rely on theory and conjecture that can be logically inferred from the results, though not conclusively shown. It is therefore important that stereotypy be looked at in a future experiment, not only to assess these behaviours for their

own sake, but also to be able to factor them out of the anxiety-related measures, or to at least make more informed statements about the potential for confounded results.

There is a large 'jump' in the mephedrone doses used in this experiment, from 1mg/kg to 20mg/kg. This was necessary to cover the full range of doses examined in only 5 different experimental groups, though conceivably there is information 'lost' in the gap between 1 and 20 mg/kg. Given that the pattern of responding is the primary feature of a dose-response relationship, it will be important to examine an intermediate dose or doses in a future study.

As discussed earlier, the defecation measure in the open field may not be a valid measure of emotionality in the current study due to the time spent in the holding cage (between injection and behavioural testing), where changes in this behaviour went unrecorded. If a future experiment required the same methodology of drug administration, counting faecal boluses both in the testing apparatus and in the holding cage could mitigate this limitation.

The novel object recognition task may have been confounded by stereotypy behaviours that appear similar to exploratory ones (e.g., head swaying in proximity to an object can be hard to distinguish from sniffing or 'exploring' the object). One way to potentially remedy this in a more specific, though less sensitive observation, might be to record only contact with an object as indicative of 'exploration', rather than contact plus proximity.

5.9 Conclusion

The results from Experiment 1 appear to support the first hypothesis that mephedrone would produce anxiety-like behaviours similar to other stimulant drugs. There may be partial support for the second hypothesis, that mephedrone would produce an inverted 'U' shaped dose-response curve, though this was not found universally. The experiment found a number

of interesting drug × sex interactions, most notably that on a number of measures male rats display a quadratic (inverted ‘U’ shape) dose-response relationship, whereas females display a linear one. Even more unexpectedly, this pattern of responding for males and females is then reversed on some other measures. A theory was proposed to explain these findings, which posits that (A): high doses of mephedrone result in stereotypy behaviours, and (B): stereotypy behaviours are expressed differently for male and female rats. More research is needed to examine the effects of stereotypy, and potentially add some validity to the current findings in the process.

6.0 Experiment 2 – Stereotypy

6.1 Stereotypy Overview

The term ‘stereotypy’ refers to a repetitive or ritualistic set of behaviours that appear compulsive and without any apparent purpose (Stereotyped behaviour induced by amphetamine, 1972). They are observed throughout the animal kingdom from invertebrates, to birds, to mammals, including humans (Ridley, 1994). At the lower levels of phylogeny these ‘fixed action patterns’ are thought to be a response to specific events in the environment, and once triggered, are performed until another event (often the outcome of the fixed action pattern) changes the animals’ behaviour, which in many cases is to another fixed action pattern. Some moths, for example, will instantly fold their wings and drop to the ground (fixed action pattern) in response to certain ultrasonic signals produced by bats (trigger event) (Roeder, 1975). Other examples include courtship displays, hunting, nest building, and attack or escape movements (NC State University, 2006). In humans and other higher mammalian species these fixed action patterns are often considered ‘reflex behaviours’, though our behavioural repertoires generally also extend to flexible, self-initiated, and voluntary behaviours as well. The loss of these latter abilities is an important feature of various psychopathologies, such as autism spectrum disorders, intellectual disabilities, tardive dyskinesea, Tourette’s syndrome, fronto-temporal dementia, obsessive compulsive disorder, and schizophrenia (Ridley, 1994). Stereotypy behaviours can range from simple to complex bodily movements (motor stereotypies) but can also include “repetitive, inflexible patterns of attention, emotion, planning and cognition typifying some clinical disorders in humans” (Canales & Grabiel, 2000, p. 377).

6.2 Induced Stereotypies

Stereotypy behaviours have been observed and can be induced in animals in a number of specific situations or conditions.

6.2.1 Confinement stereotypy. Also called ‘cage stereotypy’, these behaviours can occur when mammals are confined in small cages or enclosures, such as in zoos or in laboratory settings. The repetitive behaviours generally involve abnormal locomotion (e.g., pacing up and down one side of the enclosure) or ‘bar mouthing’ (the animal opens its mouth wide on the bars of their cage, and makes ‘sham’ biting movements). Confinement stereotypies are thought to result from the environment impairing the animals’ ability to perform species-specific behaviours (such as foraging, hunting, or interacting with other animals), and as such are easily broken by providing a bigger, more stimulating enclosure with access to the above opportunities (Ridley, 1994).

6.2.2 Deprivation stereotypy. These behaviours are observed in animals that have been reared in isolation, and usually consist of repetitive movements of body parts (such as rocking, head banging, or sucking fingers or thumbs). The behaviours can often be socially inappropriate (e.g., aggressive, or sexual in nature), and self-harming (Ridley, 1994). It has been suggested that unlike confinement stereotypy, which is a result of the environment, deprivation stereotypy is due to the lack of *interaction* with the environment during a critical developmental period. In humans, this may plausibly explain the high degree of motor stereotypies observed in severely autistic individuals, where their neurodevelopmental condition has impaired their ability to interact with their environment during a critical period in infancy (Ridley, 1994). Studies using rats have shown that environmental conditions during infancy can have a permanent effect on brain biochemistry, and accordingly, deprivation stereotypies are extremely difficult to disrupt (Ridley, 1994; Sahakian et al., 1975).

6.2.3 Drug-induced stereotypy. Stimulant drugs like amphetamine and cocaine have been consistently shown to produce stereotypy behaviours in laboratory animals. These drugs typically decrease the variation in observed behaviours, and produce continuous, repetitive,

hyperactive behaviours that are without apparent purpose (Stereotyped behaviour induced by amphetamine, 1972). Common examples of stereotyped behaviours followed amphetamine treatment include: head swaying/bobbing, limb movements, sniffing, licking, and biting of the home cages (Wolgin, 2012). These behaviours are not simply a result of overstimulation, as sedative drugs like benzodiazepine are unable to inhibit them, even when administered at doses that interfere with the righting reflex (Stereotyped behaviour induced by amphetamine, 1972). Stereotypy-inducing drugs appear to disrupt the normal, integrated activity of the brain, suspending goal-directed behaviours and responses to meaningful environmental stimuli (Wolgin, 2012).

6.3 Experiment 2 Aims and Hypotheses

Experiment 2 sought to examine the effects of mephedrone on anxiety-related behaviours in hooded rats, as well as quantifying the stereotypy behaviours observed in Experiment 1. Using a similar methodology to the earlier experiment, this study looked more closely at the 'active range' of mephedrone doses identified (and removed the two highest doses which produced the most adverse effects). In Experiment 1, males and females differed in their dose-response patterns of responding primarily at doses above 20mg/kg. As in the current study 20mg/kg was the highest dose, it was hypothesised that mephedrone would produce more anxiety-like behaviours as dose increased, and that the dose-response pattern would be the same for males and females.

Additionally, Experiment 2 included a number of stereotypy measures meant to assess behaviours observed in Experiment 1. Based on previous observations and on explanatory theories proposed in discussion of the previous results, it was hypothesised that mephedrone would induce more stereotypy behaviours as dose increased, and that males and females would differ in their expression of these behaviours.

7.0 Method

7.1 Subjects

The subjects used in this study were 32 male and 32 female PVG/c hooded rats bred in the Animal Facility of the Department of Psychology at the University of Canterbury, Christchurch, New Zealand. Following weaning at PND30 (Postnatal day 30) the subjects were housed in 525 x 330 x 230mm-high plastic cages in groups of four same-sexed animals. They were kept on a 12-hour light/dark cycle (lights on at 0800 hours) at an ambient temperature of 22 ± 2°C (with humidity of 48% ± 10%). All subjects had free access to standard laboratory food and drinking water at all times. The care and experimental treatment of subjects complied with Parts 5 (Code of Welfare) and 6 (Use of Animals in Research, Testing and Teaching) of the New Zealand Animal Welfare Act, 1999 and had been approved by the Animal Ethics Committee of the University of Canterbury (See Appendix G).

7.2 Mephedrone Treatment

The subjects were randomly assigned to one of four conditions, with each condition containing 8 male and 8 female subjects. The conditions were: 1mg/kg, 10mg/kg, or 20mg/kg of mephedrone, and a saline control group. This dose range was selected to represent the most active and interesting range found in Experiment 1 (with 10mg/kg added to observe any effects between the otherwise large jump from 1mg/kg to 20mg/kg). 40mg/kg and 60mg/kg were discontinued in this experiment due to adverse effects observed in Experiment 1. The mephedrone used in this study was synthesised on-site in the Department of Psychology at the University of Canterbury. The resulting mephedrone 98% salt was dissolved daily in isotonic saline to create solutions of the various dosages. These doses were then administered to the subjects via intraperitoneal (I.P.) injections in a volume of 1ml/kg.

At approximately PND100 (adulthood) all subjects were treated once per day, on two non-consecutive days. The subjects were removed from their home cage, injected with the

appropriate solution (with respect to their assigned experimental condition), and then placed in their own separate, covered 'holding cage'. Twenty minutes post-injection (when the drug, if administered, had taken effect) the subjects were removed from their 'holding cage' and placed in the appropriate testing apparatus (see '7.3 Behavioural Testing' section below). Following testing, subjects were returned to their home cages.

7.3 Behavioural Testing

Behavioural testing occurred 20 minutes following drug treatment (one test per day, on two non-consecutive testing days). All subjects were tested using two behavioural testing paradigms: the open field (OF), and the elevated plus maze (EPM). The order in which the subjects experienced the two behavioural tests was counterbalanced to account for any potential order or learning effects, and the two testing days were always separated by a rest day. All tests were performed in one of two experimental rooms under low-light conditions.

Immediately following each individual subject's test, the apparatus was thoroughly cleaned with a Sani Express 4% solution to attenuate confounding odour cues from previous trials. Further to this, on the days when both males and females were tested in the same apparatus, males were always tested first. This was because it has been suggested that males may be particularly sensitive to odour cues left behind by females even after thorough cleaning (Hughes, 2007).

All tests were observed via a small CCTV camera suspended over the apparatus. This allowed the experimenter to view the subjects from a television monitor some distance away in the room (i.e., so they weren't standing over the apparatus) and reduced the likelihood of their presence affecting the subjects' behaviour. The footage of each trial was recorded to a hard drive for later examination.

7.3.1 Open Field Testing. The open field apparatus and procedure were replications of those described for Experiment 1 (See 3.3.1 Open Field – Apparatus, and 3.3.2 Open Field – Procedure). The only notable differences were that the trials were not coded live but rather recorded for later observation. This was due to the addition of a number of behavioural measures that necessitated multiple viewings of the recordings. The same anxiety-related measures recorded in Experiment 1 were again observed, including: ambulation, walking, rearing, grooming, freezing, corner occupancy, and center occupancy (defecation was omitted from this experiment). In addition, using a modified version of the Canales and Grabiel (2000) procedure, four measures of stereotypy were also coded and assessed: head swaying, gyration, thigmotaxis, and stereotypy total. 'Head swaying' represented the uniform and repetitive swaying of the head, from left to right (a behaviour visually distinct from the 'normal' head movements associated with smelling and exploration). 'Gyration' involved the subjects turning in tight, full circles, often repetitively (i.e., spinning on the spot). 'Thigmotaxis' was a measure of the subjects' tendency to trace the outside of the apparatus, moving along the edges of the open field and continuing to follow the line of the 'walls'. It was thought that high levels of thigmotaxis might potentially confound other measures such as corner occupancy. The final measure, 'stereotypy total', was a composite score comprised of both head swaying and gyration (thigmotaxis was excluded as it proved a difficult behaviour to quantify as distinct from 'normal exploration'). As with the procedure outlined for the anxiety-measures, every 3 seconds the experimenter recorded which (if any) of these stereotypy measures the subjects were engaging in. It was then possible to calculate the relative time spent engaging in these behaviours over the 5 minutes of testing.

7.3.2 Elevated Plus Maze Testing. Similar to the open field testing described above, the elevated plus maze apparatus and procedure were the same as for Experiment 1 (see 3.3.3 Elevated Plus Maze – Apparatus, and 3.3.4 Elevated Plus Maze – Procedure) apart from

recording of the trials for later coding, and the inclusion of stereotypy measures. Percent entries into the open arms, percent observations in the open arms, and number of entries into the closed arms were recorded, as were head swaying, gyration, and stereotypy total (thigmotaxis was not included for this test as the apparatus lacked a clear 'boundary' for the subjects to follow).

7.4 Non-Behavioural Measures

In addition to the behavioural measures each subject's weight and temperature were also recorded. Weight, in grams, for each subject was measured using standard laboratory scales on both of the two testing days (necessary to calculate the appropriate volume of solution to inject). The weight on the first day was subtracted from the weight on the second day to give a figure representing the change in weight for that subject over the three days of testing. It was then possible to compare weight change over this time between the different experimental groups.

The subjects' temperature was also recorded, using a Braun IRT 4020 ThermoScan aural thermometer. Temperature recordings were taken on both testing days for all subjects. The first recording was taken at time of drug (or saline) administration, and the second was taken immediately following behavioural testing (i.e., 25 minutes following drug administration). The first temperature was subtracted from the second to give a figure representing the change in temperature, and this difference score was averaged for each subject across both testing days. The final figure then was an average temperature change (in degrees Celsius) for each subject, and allowed for comparisons to be made between different experimental groups.

8.0 Results

Unless otherwise stated, all measures were statistically analysed using separate 4 (drug) × 2 (sex) ANOVAs, and Fisher PLSD post hoc comparisons ($p<0.05$). Where appropriate, ANCOVAs were also utilised in order to test the significance of a given result after accounting for a potentially confounding co-variable. All analyses were performed using Statistica 12 software by StatSoft©.

No animals required exclusion from testing or analysis, so the data from all 64 subjects were used in the various statistical analyses.

8.1 Open Field Results – Anxiety Measures

The effects of mephedrone on the various anxiety measures in the open field are presented in Figure 9. As can be seen, as the mephedrone dose increased: ambulation and walking behaviours increased; while rearing, freezing, and grooming behaviours decreased. Mephedrone appears to have increased center occupancy as well, with this increase in behaviour peaking at 10mg/kg. Corner occupancy shows the inverse, with mephedrone decreasing the frequency of the behaviour most notably at 10mg/kg. The effects of mephedrone on center and corner occupancy remained significant after controlling for ambulation as a co-variable [$F(3,58) = 6.20$, $p<0.01$; and $F(3,58) = 4.62$, $p<0.01$ respectively].

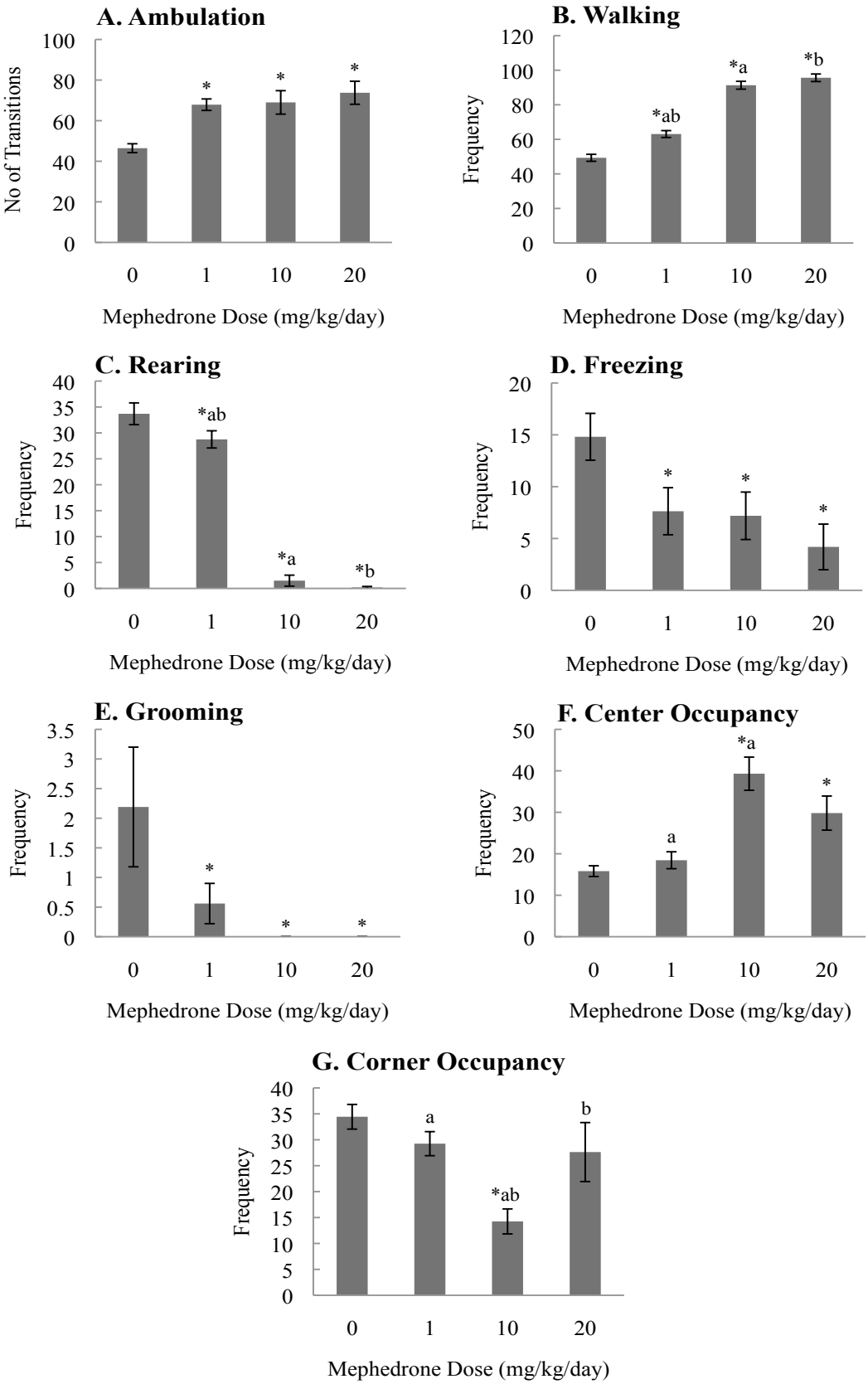

Figure 9. Mean ± S.E.M. frequencies for (A) ambulation, (B) walking, (C), rearing, (D) freezing, (E) grooming, (F) center occupancy, and (G) corner occupancy recorded in the open field following treatment with mephedrone. Data represents results for both male and female rats combined.
*Significantly different from control group ($p < 0.05$).
[a,b,c]Groups with superscripts in common are significantly different ($p < 0.05$).

8.2 Open Field Results – Stereotypy Measures

Four stereotypy measures observed in the open field are presented in Figure 10. As can be seen, mephedrone significantly increased the frequency of all four measures. 1mg/kg did not significantly increase either the head swaying or gyration measures separately, though when combined in the stereotypy total measure even this low dose creates a significant effect.

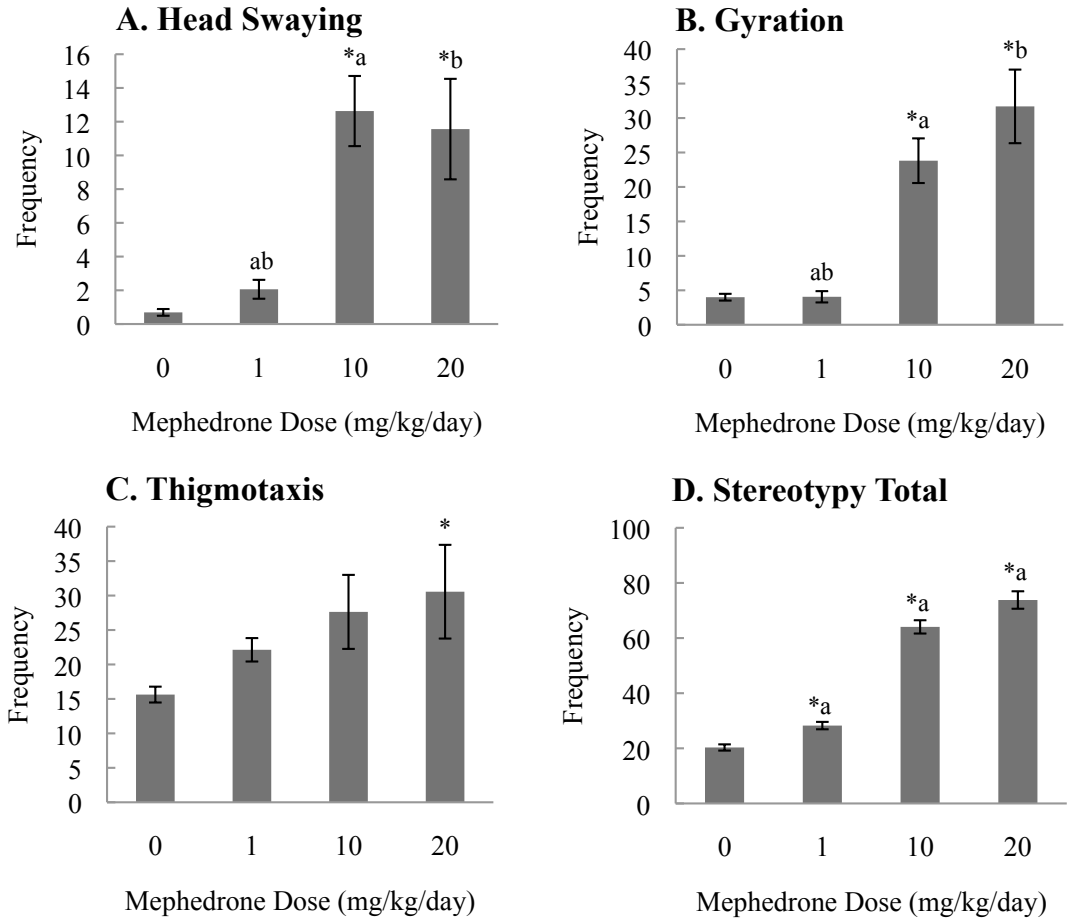

Figure 10. Mean ± S.E.M. frequencies for (A) head swaying, (B) gyration, (C), thigmotaxis, and (D) stereotypy total recorded in the open field following treatment with mephedrone. Data represents results for both male and female rats combined.
*Significantly different from control group ($p < 0.05$).
[a,b,c]Groups with superscripts in common are significantly different ($p < 0.05$).

After controlling for ambulation as a co-variable, the effects of mephedrone remained significant for head swaying [$F(3,58) = 38.62$, $p<0.001$], gyration [$F(3,58) = 22.17$, $p<0.001$], and stereotypy total [$F(3,58) = 131.09$, $p<0.001$]; whereas thigmotaxis was no longer significant [$F(3,58) = 0.79$, $p=0.50$].

The stereotypy total measure had a significant drug × sex interaction, $F(1,56) = 5.11$, $p < 0.01$. Figure 11 shows that male and female subjects' stereotypy total scores both appeared to have increased correspondingly up to 10mg/kg of mephedrone. At 20mg/kg the males' scores appear not to have changed, whereas the females' continued to increase.

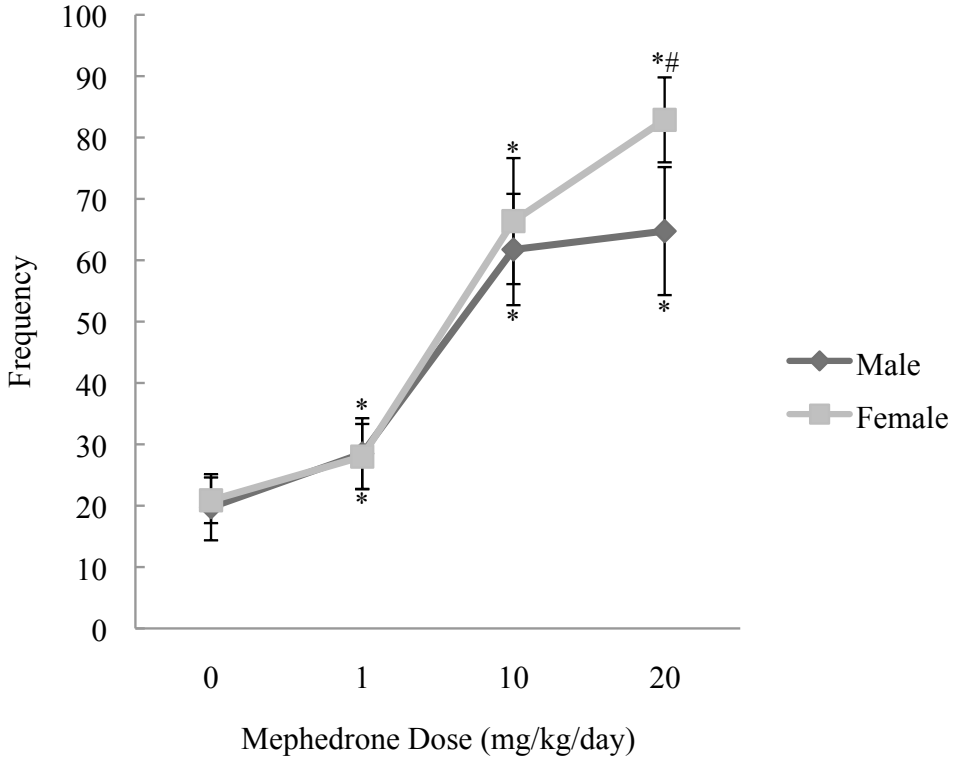

Figure 11. Mean ± S.E.M. frequencies for males and females on the stereotypy measure in the open field following treatment with four doses of mephedrone.
*Significantly different from control group for that sex ($p < 0.05$).
Signifies a significant difference between males and females for that dose ($p < 0.05$).

8.3 Elevated Plus Maze Results – Anxiety Measures

Three measures of anxiety in the elevated plus maze are displayed in Figure 12. As can be seen, mephedrone decreased percent observations in and entries into the open arms of the maze, though this effect is only significant for 20mg/kg. None of the doses of mephedrone had any significant effect on the number of entries into the closed arms.

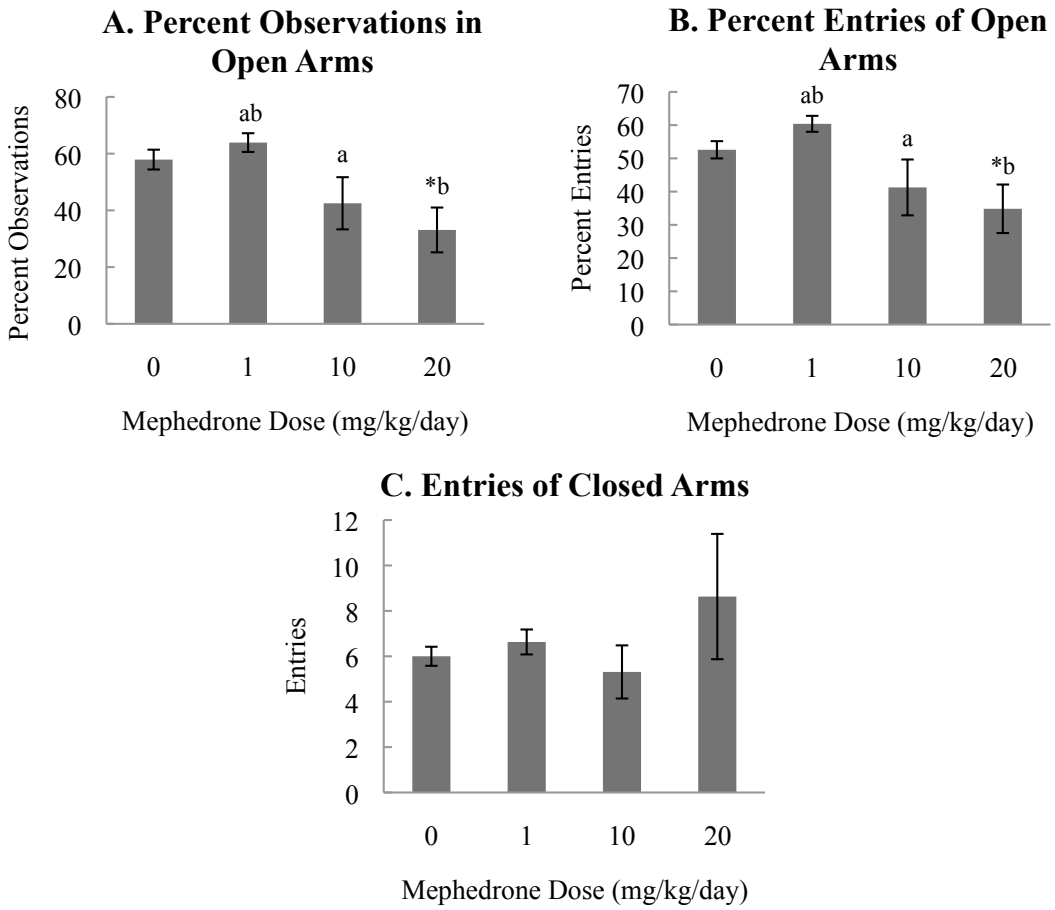

Figure 12. Mean ± S.E.M. frequencies for (A) percent observations in the open arms, (B) percent entries into the open arms, and (C) entries into the closed arms of the elevated plus maze following treatment with mephedrone. Data represents results for both male and female rats combined.
*Significantly different from control group ($p < 0.05$).
[a,b,c]Groups with superscripts in common are significantly different ($p < 0.05$).

8.4 Elevated Plus Maze – Stereotypy Measures

Similar to measures in the open field; head swaying, gyration, and stereotypy total were recorded in the elevated plus maze as measures of stereotypy. Figure 13 shows that as mephedrone dose increased, all three of these behavioural measures increased in frequency. Head swaying and gyration showed no significant effect at 1mg/kg, though when combined in the stereotypy total measure this low dose was significantly different from the control group.

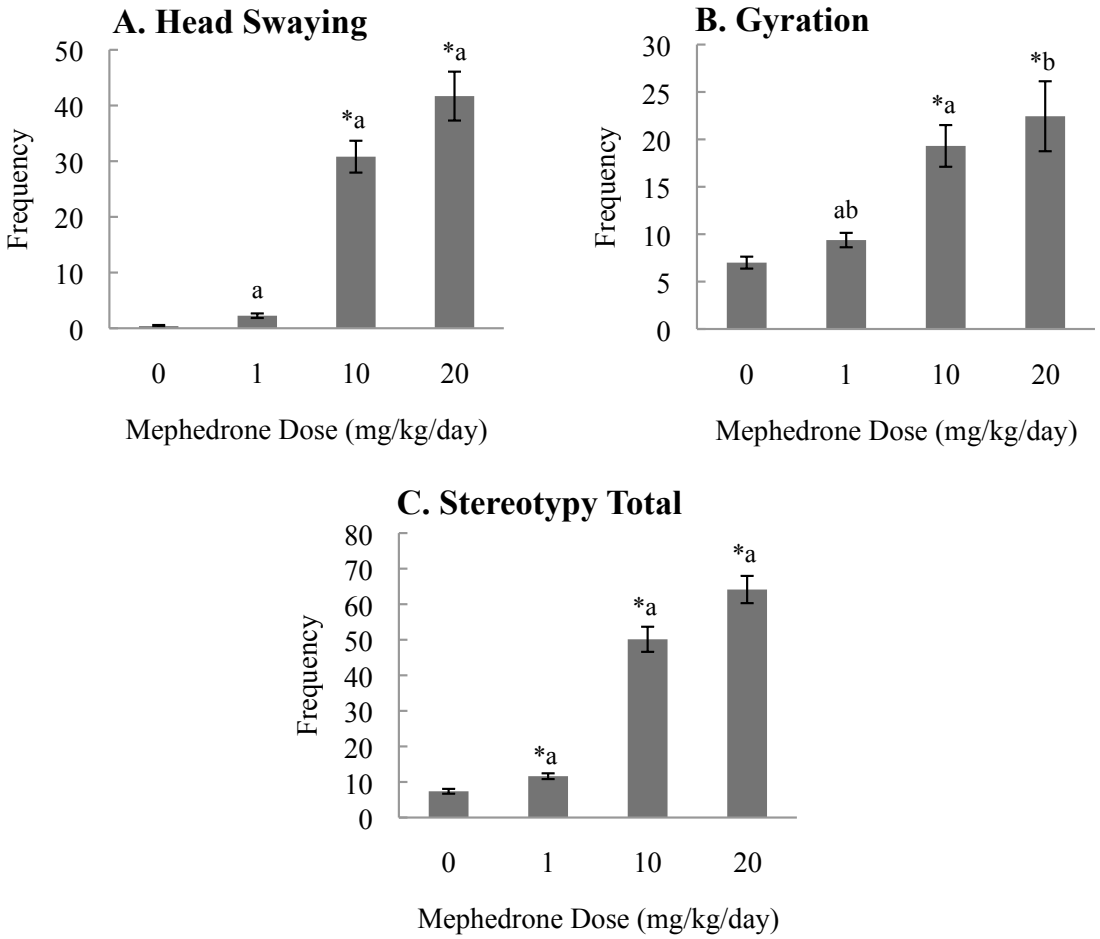

Figure 13. Mean ± S.E.M. frequencies for (A) head swaying, (B) gyration, and (C) stereotypy total recorded in the elevated plus maze following treatment with mephedrone. Data represents results for both male and female rats combined.
*Significantly different from control group ($p < 0.05$).
[a,b,c]Groups with superscripts in common are significantly different ($p < 0.05$).

8.5 Sex Differences

The overall sex differences for each of the behavioural measures examined are presented in Table 3. As can be seen, in the open field female subjects travelled significantly further distances (ambulation) than males, froze less, and displayed more total stereotypy behaviours. In the elevated plus maze, females spent significantly more time than males stereotypically turning in circles (gyration). On the measures that did not reach statistical significance, most of the results suggest that females generally performed those behaviours more frequently than males.

Table 3
Means, S.E.Ms, and ANOVA results for main effects of Sex on all measures.

	Sex of Rats		
Measure	Male	Female	F(1,56)
Open Field			
Ambulation	58.00(±3.41)	70.53(±3.45)	8.98**
Walking	74.09(±3.73)	75.56(±3.80)	0.48
Rearing	14.75(±2.74)	17.31(±3.09)	3.28
Freezing	10.69(±1.88)	6.22(±1.43)	4.12*
Grooming	0.47(±0.25)	0.91(±0.51)	0.66
Centre Occupancy	26.06(±2.90)	25.62(±3.50)	0.01
Corner Occupancy	25.66(±2.80)	27.13(±2.76)	0.17
Head Swaying	8.22(±1.79)	5.25(±1.34)	2.89
Gyration	15.44(±2.54)	16.34(±3.57)	0.08
Thigmotaxis	20.03(±2.38)	27.94(±3.82)	3.29
Stereotypy Total	43.69(±3.80)	49.53(±4.80)	9.74**
Elevated Plus Maze			
% Entries in Open Arms	*0.45(±0.04)*	*0.50(±0.04)*	*0.70*
% Observations in Open Arms	*0.46(±0.05)*	*0.53(±0.05)*	*1.26*
Entries into Closed Arms	5.16(±0.43)	8.13(±1.44)	3.90
Head Swaying	20.00(±3.65)	17.56(±3.74)	0.83
Gyration	11.25(±1.13)	17.81(±2.33)	11.54**
Stereotypy Total	31.25(±4.30)	35.38(±5.13)	2.44

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

8.6 Non-Behavioural Measures

The average difference in the subjects' weight between the two testing days is displayed in Figure 14. The results suggest that subjects receiving 1mg/kg of mephedrone gained significantly more weight than control rats, as well as more than those administered higher doses. Those subjects administered 10mg/kg and 20mg/kg showed no significant differences in weight change from control animals.

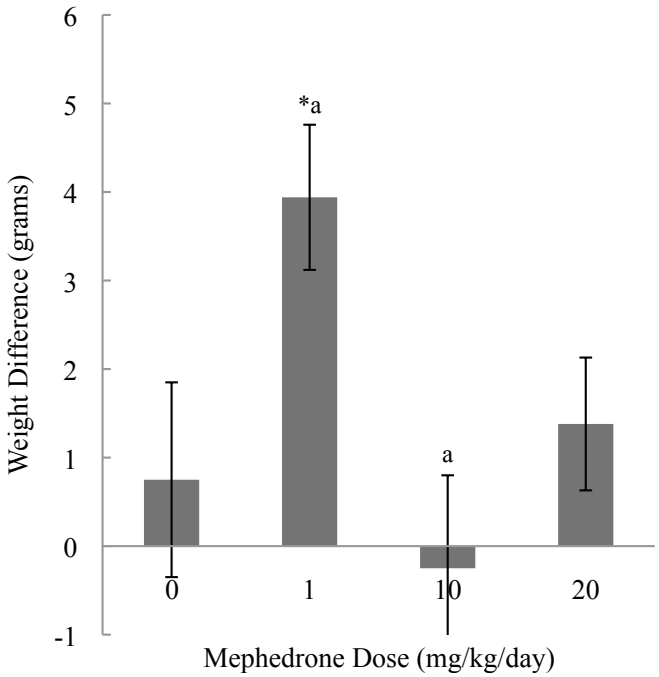

Figure 14. Mean ± S.E.M. results for weight difference (in grams) over the 3 days of testing. Data represents results for both male and female rats combined.
*Significantly different from control group ($p < 0.05$).
[a,b,c]Groups with superscripts in common are significantly different ($p < 0.05$).

The changes in each subjects' temperature 20 minutes following drug administration were recorded and averaged across the two testing days. Figure 15 displays the average difference across experimental groups. As can be seen, the 20mg/kg group showed a significantly greater increase of temperature than the 1mg/kg and 10mg/kg groups, though none were significantly different from the control group.

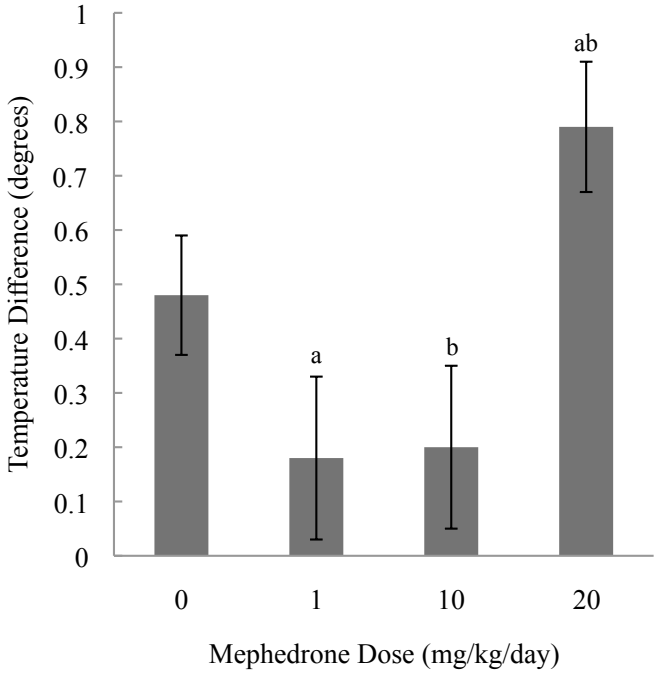

Figure 15. Mean ± S.E.M. results for average temperature difference (in degrees Celsius), 20 minutes following treatment with mephedrone. Data represents results for both male and female rats combined.
*Significantly different from control group ($p < 0.05$).
[a,b,c]Groups with superscripts in common are significantly different ($p < 0.05$).

9.0 Discussion

Experiment 2 examined the anxiety-like behaviours of male and female rats following acute treatment with 1, 10, or 20mg/kg of mephedrone, or isotonic saline. In addition, stereotypy behaviours were assessed in both the open field and elevated plus maze tests. The results generally supported Hypothesis 1; that mephedrone would produce more anxiety-like behaviours as dose increased, and in a pattern similar for both male and female subjects. The stereotypy measures also supported Hypothesis 2; that mephedrone would produce more stereotypy behaviours as the dose increased, and that males and females would express these behaviours differently.

9.1 Open Field Findings

9.1.1 Anxiety-like behaviours. Mephedrone produced a significant increase in ambulation and walking behaviours in the open field, consistent with results seen in Experiment 1, and likely a result of the direct psychostimulant effects of mephedrone. Treatment with mephedrone also resulted in decreased rearing, and grooming behaviours, which are both indicative of increased anxiety. These decreases trended down as the mephedrone dose increased, displaying a dose-dependent anxiogenic effect consistent with the first part of Hypothesis 1.

Mephedrone also significantly decreased the frequency of freezing behaviours in the open field. Decreased freezing is usually indicative of less anxiety, so this finding is at odds with the other measures and the predicted outcome. One explanation for this result is that the stimulant effects of mephedrone increased general locomotor activity, a theory supported by the results of the ambulation and walking measures, as well as previous research into similar drugs (e.g., Gatch et al., 2015; Sahakian et al., 1975). This general hyperactivity then would inherently result in less freezing behaviours or standing still, as the animals spend a greater proportion of the observation time moving about the apparatus.

The center and corner occupancy measures also had unexpected results in the open field. If mephedrone produces a dose-dependent anxiogenic effect as predicted by Hypothesis 1 (and supported by the results of other behavioural measures) we would expect to see center occupancy trending downwards and corner occupancy trending upwards with higher doses. Instead, the results for center occupancy show an increase at 10mg/kg, then a decrease at 20mg/kg. This finding is unlikely to be an artefact of the data as it is perfectly mirrored in the corner occupancy results as well (a decrease at 10mg/kg, followed by an increase at 20mg/kg).

There are really two elements to these findings that need to be explained: (A) why did center occupancy increase (and corner occupancy decrease) at 10mg/kg, which was counter to our predictions? And (B) why then did this increase (and decrease, respectively) lessen at 20mg/kg? The second element (B) may be easier to answer with the current data: the stereotypy results from the open field (discussed in the next section) show that the frequency of all stereotypy behaviours increased with higher doses of mephedrone. In particular, thigmotaxis (running along the walls of the apparatus, 'tracing' the outside edges) was significantly increased at the highest dose. This suggests that those subjects treated with 20mg/kg spent more time than subjects treated with lower doses running around the outside of the open field, which would both decrease their chances of being observed in the center squares and increase the chance of being observed in the corners. Whatever factor produced the increase and decrease in center and corner occupancy (respectively) at 10mg/kg can then be thought of as being confounded by stereotypy at 20mg/kg.

How then do we explain the initial increase and decrease (A)? Three explanations that may individually or in combination, plausibly explain the current findings are therefore suggested: 1) as with the explanation for (B), stereotypy may have confounded these measures. At 10mg/kg thigmotaxis was not significantly increased, but gyration

(turning/running in tight circles) was. Subjects gyrating over the area of two or more grid squares would be much more likely to be observed sometimes occupying a center square, particularly as the low corner occupancy and thigmotaxis scores suggest that said gyration was not frequently occurring near the corners or sides of the apparatus. 2) While 1mg/kg may not be psychoactive enough to produce a given effect, and 20mg/kg may be 'too active' (i.e., induces stereotypy behaviours significant enough to confound attempts to measure the effect), perhaps 10mg/kg is in the 'Goldilocks' zone and allows us to observe an effect of mephedrone that would otherwise be missed (i.e., by Experiment 1, which had no dose between 1 and 20mg/kg). The increase in center occupancy (and decrease in corner occupancy) could then be explained by other known or suspected effects of mephedrone, such as: disinhibition/increased risk taking, increased locomotor activity, elevated mood, or confusion. 3) A final explanation for the findings is simply that mephedrone may produce different effects, not simply different *degrees* of the same effect, at different doses (e.g., an anxiolytic effect at low doses, and an anxiogenic effect at higher doses). This latter explanation is difficult to substantiate with the current data, though is plausible and supported by previous research that has found similar synthetic cathinones to have different effects at different doses (Coppola & Mondola, 2012b; Zourob, 2011).

9.1.2 Stereotypy behaviours. Mephedrone produced a significant increase in all four stereotypy measures examined in the open field. Head swaying, gyration, thigmotaxis, and stereotypy total (a composite of head swaying and gyration measures) all display a trend of increasing frequencies of stereotypy behaviours as mephedrone dose increases, supporting the first part of Hypothesis 2. In all of the measures except the stereotypy composite, 1mg/kg did not significantly increase the frequency of the behaviours relative to control. This suggests that mephedrone does not universally elicit observable stereotypy behaviours, but

rather that there is likely a ‘threshold’ dose after which these behaviours are present and can be reliably measured.

A significant dose × sex interaction was found on the stereotypy total measure. Up to 10mg/kg both males and females displayed the same pattern of stereotypy behaviours (i.e., a dose-dependent increase) though at 20mg/kg females’ stereotypy behaviour increased further, while male stereotypy levels plateaued. This suggests that at higher doses of mephedrone the observable stereotypic effects are different for males and females, supporting the second part of Hypothesis 2.

9.2 Elevated Plus Maze Findings

9.2.1 Anxiety-like behaviours. Mephedrone produced a significant decrease in percentage entries into the open arms of the elevated plus maze, and also in the percentage time spent in the open arms. These results are indicative of increased anxiety and are consistent with results reported in other studies examining similar psychoactive compounds (Cruz et al., 1994; Martinez et al., 2002; Pellow et al., 1985; Silva & Brandao, 2000). The decrease in both measures observed with mephedrone treatment is more pronounced at higher doses of the drug (i.e., there is a dose-dependent decrease), supporting the first part of Hypothesis 1. While these findings are consistent with our predictions (based on previous research), they are different to those found in Experiment 1 (which detected no significant differences in the EPM). It is unclear at this time why this particular test produced different findings in the current experiment, particularly as the methodology was replicated exactly.

Entries of closed arms were recorded as a control measure to assess general locomotor activity, uncontaminated by anxiety (Cruz et al., 1994). There were no significant differences in the number of entries into the closed arms of the maze with the various mephedrone doses tested. What this tells us is that the results discussed for the two anxiety-related measures are

unlikely to have been contaminated by general locomotor activity (e.g., simply entering more maze arms overall due to hyperactivity).

9.2.2 Stereotypy behaviours. As in the open field results mephedrone produced a significant increase in all of the stereotypy measures recorded in the elevated plus maze. Head swaying and gyration (thigmotaxis was not recorded in the EPM) both increased in frequency as the mephedrone dose increased, as did the stereotypy total measure (the latter being unsurprising as this measure is a composite of the other two). Taken together, these results support the first part of Hypothesis 2.

9.3 Sex Differences

9.3.1 Anxiety-like behaviours. Unsurprisingly, females displayed significantly more ambulation, and less freezing behaviours than males. These findings are consistent with the known general activity differences between the sexes, and with results seen in Experiment 1. None of the other anxiety-related measures displayed significant sex differences, suggesting that males and females performed these behaviours comparably across the range of mephedrone doses examined. Taken together these results further support the second part of Hypothesis 1.

9.3.2 Stereotypy behaviours. In the open field, females were significantly higher on the stereotypy total measure than males, which is perhaps surprising given that the there were no significant differences in the two measures that comprise this composite score (head swaying and gyration). While the differences were not significant, females displayed more of the ‘active’ stereotypy behaviours than males (i.e., more gyration, less head swaying) and perhaps these differences were amplified when composited into the stereotypy total score. In the elevated plus maze females exhibited significantly more gyration than males, whereas there was no significant difference on the head swaying measure. The stereotypy total measure was similarly non-significant, likely as a result of the head swaying measure

compromising statistical power of the composite score. Taken together these results tend to support the second part of Hypothesis 2 that males and females would differ in their *expression* of stereotypy. It appears that generally females express their stereotypy in more 'active' forms than do males, within the current study.

9.4 Non-Behavioural Measures

Over the three days of testing, those subjects treated with 1mg/kg of mephedrone gained significantly more weight than control animals, and also more than those subjects on higher doses of mephedrone. It is unclear why the low-dose subjects saw this increase in weight gain; it could be that mephedrone impacts appetite or satiety, and that it does so differently at different doses. This would be an interesting area for future investigation.

The difference in the aural temperature of subjects between the time of drug injection and the completion of testing (i.e., 25 minutes later) was averaged for each subject to give the temperature difference measure. The results showed no significant differences from control for any of the doses of mephedrone. The lack of findings here may reflect measurement error, or natural variation in the data. Relative to Experiment 1 (which found a significant dose-dependent increase in temperature) the current study had smaller sample sizes (n=8, compared to n=10 from Experiment 1) and fewer tests to average the difference scores across (2 tests compared to 4 in Experiment 1). It is possible that these methodological differences contributed to the current study lacking significant statistical power to detect an effect of temperature difference.

9.5 Limitations and Future Research

As with in Experiment 1, the presence of stereotypy is still a significant limitation in this study to accurately measuring anxiety-like behaviours. Stereotypy behaviours appear to confound some behavioural measures, particularly at higher doses. This study quantified several of these stereotypy behaviours, so it was at least possible to discuss the anxiety-like

behaviours with regard to the stereotypy measures, lending some additional validity and evidence to the conclusions drawn both here and in Experiment 1. Given the apparent strong association between anxiety and stereotypy it may not be possible to separate these two in behavioural testing, in which case it will be important for future research to similarly account for the potential confound when interpreting their findings.

The current study had a sample size of 8 subjects per experimental group, whereas for Experiment 1 this sample size was 10. A sample size of 8 should provide sufficient statistical power to detect an effect of treatment, as has been successfully used in previous studies employing similar methodologies (e.g., Anderson & Hughes, 2008; Thompson, 2012), though it stands to reason that the statistical power of Experiment 1 would have been comparatively greater, so this should be factored in to any understanding of differences in findings between the two studies.

The current study added a 10mg/kg dose of mephedrone to fill a 'gap' not measured in Experiment 1. This decision was further justified when the results showed there were a number of effects found 'uniquely' at 10mg/kg that Experiment 1 had not detected. It appears as if there may be two critical 'thresholds' with increasing mephedrone doses: (1) the point at which stereotypy behaviours begin and can be reliably observed/recorded, and (2) the point at which these stereotypy behaviours are significant enough that they potentially confound anxiety-related measures. The current study suggests that threshold (1) is likely to be near 10mg/kg, and threshold (2) is likely to be near 20mg/kg. It would be interesting for future research to examine more doses between 1 and 20mg/kg to see if these 'thresholds' can be observed and more accurately defined.

Both Experiments 1 and 2 have established evidence that mephedrone produces anxiety-like behaviours following acute treatment. Future studies should also examine any potential chronic effects, both: the effects of chronic mephedrone treatment on anxiety-like

behaviours, and the long-term effects of acute mephedrone treatment (i.e., are there any lasting effects on behaviour observable later in life). In particular, does mephedrone produce any behavioural teratological effects during specific developmental periods such as adolescence (the period during which humans are most likely to begin experimenting with drugs and alcohol)?

9.6 Conclusion

The results from Experiment 2 appear to support both parts of Hypothesis 1 that mephedrone would have a dose-dependent anxiogenic effect, and that this effect would be similar for males and females. The findings also support both parts of Hypothesis 2 that mephedrone would have a dose-dependent effect on stereotypy behaviours, and that this effect would be different for males and females. The results seem to suggest that the dose-response pattern for mephedrone may have two 'thresholds': the point at which stereotypy behaviours begin, and the point at which stereotypy confounds other measures. Given that stereotypy may be impossible to separate from anxiety in behavioural tests, understanding the relationship between the two is an important factor for interpreting the results of these studies or other similar research in the future.

10.0 Experiment 3 – Adolescent Teratology and Environmental Enrichment

10.1 Teratology Overview

Teratology is a field of study that examines the causes, mechanisms, and patterns of abnormal development (Ujhazy, Mach, Navarova, Brucknerova, & Dubovicky, 2012). Exposure to certain drugs, chemicals, viruses, bacteria, parasites, radiation, and maternal stress (i.e., 'teratogens') during critical developmental periods can disrupt normal development and result in growth retardation, delayed mental development, or other structural malformations (Ujhazy et al., 2012; Wilson, 1973; Wilson & Fraser, 1977). Perhaps the best-known example of teratological outcomes in humans is the thalidomide tragedy of the late 1950's and 1960's. An immunomodulatory drug, thalidomide was sold over-the-counter to pregnant women to relieve the nausea associated with morning sickness. Shortly afterwards, thousands of infants were born with malformed limbs ('phycomelia'), eyes, and hearts, and many did not survive (Fintel, Samaras, & Carias, 2009; Miller, 1991; Ujhazy et al., 2012). Following the disaster, regulations over the development and use of drugs were significantly amended (Fintel et al., 2009; Heaton, 1994). While thalidomide was one of the more high-profile teratological events of modern times, a number of other significant events are displayed in Table 4.

Accordingly, 'behavioural teratology' refers to the study of abnormal behavioural sequelae resulting from exposure to teratogens during critical developmental periods. Disrupted growth or damage of the developing brain can have long-lasting effects on observable behaviours, as well as on physiological development.

Table 4. Significant Teratological Events in Modern History (Ujhazy et al., 2012)

Year	Teratological Event
1905	The first experimentally induced developmental toxicity in mammals. Embryonic lethality induced by X-rays in cats.
1921	The first experimentally induced teratogenicity in mammals. Disorders in limbs in pigs induced by lipid diet.
1929	He first description of malformations in humans caused by exogenous factors. Microcephalia caused by X-ray irradiation of the pelvis.
1935	Recognition of food deficiency leading to malformations in animals. Eye disorders in pigs due to hypovitaminosis A.
1937	Hormones causing alteration in sexual differentiation in animals. Masculinisation of female foetuses in mice due to the action of androgens.
1941	Report on virus-induced human malformations. Rose-rash induced eye disorders.
1944	The first evidence of postnatal effect following prenatal administration of a chemical substance. Decreased learning ability in rats caused by the administration of sodium bromide.
1948	General recognition of chemically induced teratogenicity. Experiments with alkylating agents and trypan blue.
1952	The first report on malformations caused by drugs in humans. Multiple malformations in foetuses caused by aminopterin.
1959	The first report on malformations induced by environmental pollutants. Disorders of the central nervous system and dentition caused by methyl mercury.
1961	Thalidomide-induced embryopathy

A critical factor in the susceptibility of an organism to teratogenesis is the developmental stage during which they are exposed to the teratogen. Generally, organisms are most susceptible to teratogenic influences during periods of rapid cell differentiation, growth, and development. Accordingly, in mammals the most vulnerable periods of development are the prenatal and lactational stages, as well as adolescence (Rice & Barone Jr, 2000). Exposure to teratogens during these periods can have long-lasting or permanent effects on development.

10.2 Adolescence

Adolescence can be defined as the developmental period transitioning from immaturity and dependence, to maturity and independence (Rowse, 2010; Spear, 2007). It includes, but is not synonymous with puberty and its associated physiological changes and growth spurt. Of specific interest to psychologists are the behavioural features associated with adolescence, which include: increased novelty seeking, increased risk taking behaviours, a preference for social affiliation and peer-based social interactions, and experimentation

with drugs and alcohol (Irwin Jr, 1989; La Greca, Prinstein, & Fetter, 2001; Trimpop, Kerr, & Kirkcaldy, 1999). The adolescent brain in still developing (Spear, 2007), and while the brain does not grow dramatically in size during adolescence (having reached 90% of its total adult size by age six); myelination (white matter production that increases the speed of neural signals), and synaptic pruning (the selective process whereby often-used neural connections are strengthened, while unused connections are eliminated) significantly reorganise the brain throughout this period (Casey, Tottenham, Liston, & Durston, 2005; Rowse, 2010). The timing of the adolescent period is often difficult to define. The developmental period in humans is often described as ranging from 12-18 years of age, though some features may begin as early as 8-10 (especially in females), and some may last as late as until 25 (especially in males) (Spear, 2007).

10.2.1 Adolescence in Rats. The developmental stage of adolescence is not unique to humans, with other species exhibiting comparable physiological and behaviour changes. In addition to undergoing the hormonal changes and growth spurt associated with puberty; adolescent rats seek more novelty, display greater social conditioned place preference (CPP), and are more inclined to voluntarily consume alcohol compared to adults (Spear, 2007). The timing of the adolescent period in rats is also as comparably difficult to define as it is in humans. Researchers have conservatively classified adolescence as ranging from 28 to 42 postnatal days, based on the timing of the growth spurt, puberty, and behavioural changes (Spear, 2000). However, similar to the relative differences in humans; some features of adolescence may be seen as early as postnatal day 23 (especially in females), and other signs can still be observed as late as postnatal day 55 (especially in males) (Spear, 2007). Studies involving adolescence in rats therefore need to be selective about the precise time-period examined based on the research question being studied and the species of rat being used.

10.3 Environmental Enrichment

Environmental enrichment has been shown to produce a number of positive physiological and behavioural outcomes in both humans and animals (Frick & Fernandez, 2003; Shimamura, Berry, Mangels, Rusting, & Jurica, 1995). Early exposure to enriched social, physical activity-related, and perceptual stimulation has beneficial effects on both brain and behavioural development (Renner & Rosenzweig, 1987). It has been suggested that such enrichment induces a number of neurochemical changes in the brain such as increased cortical thickness, synaptic contacts, and number of dendritic spines; resulting in greater neural plasticity and thus improved learning and memory (Rosenzweig & Bennett, 1995; van Praag, Kempermann, & Gage, 2000).

In studies using rodents, this type of enrichment is usually accomplished via the provision of objects or toys designed to encourage exploration, manipulation, and physical activity; to the home cages of group-housed subjects. Relative to rats housed in 'standard' cages, these 'enriched' rats have been shown to exhibit greater learning and memory capacity, as well as decreased anxiety-like behaviours in behavioural tests (Benaroya-Milshtein et al., 2004; Hughes & Collins, 2010; Laviola et al., 2004).

10.4 Experiment 3 Aims and Hypotheses

Experiment 3 sought to examine the teratological effects of adolescent mephedrone use on long-term anxiety-like behaviour. Previous studies have found that the use of psychomotor stimulants during adolescence produces anxiogenic effects detectable, following abstinence, later in life (e.g., Aitchison & Hughes, 2006; Anderson & Hughes, 2008; Thompson, 2012). This suggests that these types of drugs can have long-lasting or permanent effects when used specifically during critical developmental periods. The results of both Experiment 1 and 2 suggest that mephedrone has acute anxiogenic properties, but the potential for chronic effects has not yet been examined. Experiment 3 therefore involved

treatment of adolescent rats with mephedrone and then measured anxiety-like behaviours later in adulthood, with a view to identifying any lasting impacts of mephedrone exposure.

In addition, Experiment 3 also sought to examine the effects of environmental enrichment on anxiety-like behaviours, specifically if the anxiolytic effects of enrichment would mitigate any anxiogenic effects of mephedrone. If so, this information would be useful in understanding and building resistance and resilience to some of the harmful and long-lasting effects of drug use during adolescence.

It was hypothesised that those rats treated with mephedrone during adolescence would display more anxiety-like behaviours in adulthood compared to the control group (Hypothesis 1). It was also hypothesised that, overall, environmentally enriched rats would display fewer anxiety-related behaviours compared to rats reared in ‘standard’ cages (Hypothesis 2). It was further hypothesised that of the rats treated with mephedrone, those raised in ‘enriched’ environments would display fewer anxiety-like behaviours than those raised in ‘standard’ cages (Hypothesis 3).

11.0 Method

11.1 Subjects

The subjects used in this study were 32 male and 32 female PVG/c hooded rats bred in the Animal Facility of the Department of Psychology at the University of Canterbury, Christchurch, New Zealand. Following weaning at PND30 (Postnatal day 30) half of the animals (16 males and 16 females) were randomly assigned to the 'enriched' caging condition (see 11.2 Environmental Enrichment below) and the other half to the 'standard' caging condition. All subjects were housed in 550 x 360 x 220mm-high plastic cages in groups of four same-sexed animals. They were kept on a 12-hour light/dark cycle (lights on at 0800 hours) at an ambient temperature of 22 ± 2°C (with humidity of 48% ± 10%). All subjects had free access to standard laboratory food and drinking water at all times. The care and experimental treatment of subjects complied with Parts 5 (Code of Welfare) and 6 (Use of Animals in Research, Testing and Teaching) of the New Zealand Animal Welfare Act, 1999 and had been approved by the Animal Ethics Committee of the University of Canterbury (See Appendix H).

11.2 Environmental Enrichment

The subjects assigned to the enriched caging condition were provided with four different objects or 'toys' to interact with in their home-cage. These enrichment objects were chosen at random from a selection containing items such as glass jars, plastic tunnels, cups, balls, small wooden toys, household utensils, small chains, and cardboard boxes (see Figure I1 in Appendix I). The objects were chosen to promote and encourage exploration, manipulation, and physical activity (as used by Hughes & Collins, 2010; Thompson, 2012). The objects in each cage were changed once per week with another four randomly chosen from the collection. This was to help ensure that all subjects had different and 'novel' toys to interact with, and so that each cage was unlikely to receive the same selection of four objects

more than once. The subjects assigned to the 'standard' caging conditions were housed in identical cages, though were not supplied with toys or objects to interact with (see Figure I2 in Appendix I).

11.3 Mephedrone Treatment

Half of the subjects in each caging condition (8 males and 8 females) were randomly assigned to a drug treatment condition, and the other half to a saline control. Those in the drug treatment condition received mephedrone at a dose of 20mg/kg, a dose found to produce significant effects in both Experiments 1 and 2. The mephedrone used in this study was synthesised on-site in the Department of Psychology at the University of Canterbury. The resulting mephedrone 98% salt was dissolved daily in isotonic saline to create a 20mg/kg solution. These doses were then administered to the subjects via intraperitoneal (I.P.) injections in a volume of 1ml/kg.

Beginning on PND45 the subjects were treated with either mephedrone or isotonic saline (for the control group) once per day, for 10 consecutive days. PND45 to PND55 is a developmental period that has been used previously in similar studies to approximate human adolescence in rats (Aitchison & Hughes, 2006; Anderson & Hughes, 2008; Thompson, 2012), and this pattern of treatment was designed to represent chronic use during this time.

11.4 Behavioural Testing

Behavioural testing began when the subjects reached adulthood at approximately PND110. All subjects were tested once per day, using three behavioural testing paradigms: the open field, the light/dark box, and the elevated plus maze. The order in which the subjects experienced the three behavioural tests was counterbalanced to account for any potential order or learning effects, and the three testing days were always separated by a 'rest' day. All tests were performed in one of two experimental rooms under low-light conditions.

Immediately following each individual subject's test, the apparatus was thoroughly cleaned with a Sani Express 4% solution to attenuate confounding odour cues from previous trials. Further to this, on the days when both males and females were tested in the same apparatus, males were always tested first. This was because it has been suggested that males may be particularly sensitive to odour cues left behind by females even after thorough cleaning (Hughes, 2007).

All tests were observed via a small CCTV camera suspended over the apparatus. This allowed the experimenter to view the subjects from a television monitor some distance away in the room (i.e., so they weren't standing over the apparatus) and reduced the likelihood of their presence affecting the subjects' behaviour.

11.4.1 Apparatus' and Procedure. The open field, light/dark box, and elevated plus maze and procedures were the same as for Experiment 1 (See 3.3 Behavioural Testing). For the open field, the same anxiety-measures recorded in Experiment 1 were again observed, including ambulation, walking, rearing, grooming, freezing, corner occupancy, and center occupancy (as with Experiment 2, defecation was omitted from this experiment). In the light/dark box; observations in the light side, entries into the light side, first emergence latency, and number of prior emergences were recorded. In the elevated plus maze, percent observations in the open arms, percent entries into the open arms, and number of entries into the closed arms were all recorded. Stereotyped behaviours were not assessed in this experiment, as the testing occurred weeks after drug administration rather than immediately afterwards (as with Experiments 1 and 2) where acute drug effects (like stereotypy) might be observed.

11.5 Non-Behavioural Measures

In addition to the behavioural measures, each subject's weight at adolescence and at adulthood was also recorded. Weight, in grams, for each subject was measured using

standard laboratory scales on each of the ten treatment days (necessary to calculate the appropriate volume of solution to inject) as well as on the final testing day. The average weight during treatment (‘adolescence’) was subtracted from the final weight (‘adulthood’) to give a figure representing the change in weight for that subject over the two months between treatment and testing. It was then possible to compare weight gain over this time between the different experimental groups.

12.0 Results

Unless otherwise stated, all measures were statistically analysed using separate 2 (drug) × 2 (enrichment) × 2 (sex) factorial ANOVAs, and Fisher PLSD post hoc comparisons ($p<0.05$). Where appropriate, ANCOVAs were also utilised in order to test the significance of a given result after accounting for a potentially confounding co-variable. All analyses were performed using Statistica 12 software by StatSoft©.

Subject number 20 (male, enriched, saline control) was found dead in its cage between the treatment and testing phases of the experiment. The death was deemed not to be connected to the experiment, and none of its cage-mates showed any abnormal behaviours or signs. Nevertheless, as it died before the testing phase it was clearly not able to be included in the data set.

12.1 Open Field Results

The main effects for both drug treatment and enrichment condition, along with means and standard errors for all of the open field measures are displayed in Table 5. As can be seen, the subjects treated with mephedrone during adolescence displayed significantly less ambulation and rearing behaviours, and more freezing behaviours compared to saline controls. Mephedrone's effect on walking and grooming behaviours was negligible, as it was also for occupancy of the center or corner squares, with these measures failing to show any significant differences. Environmental enrichment significantly decreased the frequency of walking and freezing behaviours, and increased the frequency of rearing. Ambulation appeared to be decreased in the enriched relative to standard condition, although this difference was not statistically significant. Grooming, center occupancy, and corner occupancy all again showed negligible differences between the two caging conditions.

Table 5
Means, S.E.Ms, and ANOVA results for effects of Drug and Enrichment on all OF measures.

	Drug			Enrichment		
Measure	Saline	Mephedrone	F(1,55)	Standard	Enriched	F(1,55)
Ambulation[a]	48.29(±1.94)	40.59(±1.92)	10.46**	46.06(±2.24)	42.65(±1.77)	2.46
Walking	46.19(±1.48)	45.59(±1.13)	0.07	49.25(±1.18)	42.42(±1.13)	18.80***
Rearing[b]	41.85(±1.82)	33.97(±2.18)	10.81**	31.50(±1.78)	43.90(±1.81)	29.41***
Freezing[c]	8.48(±1.50)	16.06(±1.97)	11.18**	15.19(±1.86)	9.39(±1.76)	6.65*
Grooming	3.97(±0.49)	4.38(±0.43)	0.32	4.06(±0.38)	4.29(±0.53)	1.02
Centre Occupancy	10.06(±0.92)	10.00(±1.20)	0.00	9.84(±1.11)	10.23(±1.03)	0.05
Corner Occupancy[d]	48.45(±2.06)	48.59(±2.41)	0.00	48.75(±2.26)	48.29(±2.24)	0.01

*p < 0.05, **p < 0.01, ***p < 0.001
[a]Drug × Enrichment interaction significant, $F(1,55) = 18.83$, $p < 0.001$
[b]Sex × Enrichment interaction significant, $F(1,55) = 4.07$, $p < 0.05$
[c]Drug × Enrichment interaction significant, $F(1,55) = 5.72$, $p < 0.05$
[d]Drug × Enrichment interaction significant, $F(1,55) = 7.11$, $p < 0.05$

As Table 5 indicates, there were a number of significant interactions found in the open field measures. These are displayed graphically in Figure 16.

As can be seen in (A), mephedrone significantly decreased ambulation in the open field for the standard caging condition, but not for the enriched condition. Similarly for freezing (C), mephedrone produced a significant increase in this behaviour in the standard caging but this effect was not found for the enriched condition. Corner occupancy (D) appeared to increase with mephedrone treatment for those in standard cages, whereas those in enriched cages showed a slight decrease in this behaviour when treated with the drug (though neither of these main effects were significant). The frequency of rearing in the open field (B) was different for males and females in different caging conditions. Female, standard subjects reared more often than male, standard subjects; whereas enriched males and females showed no significant difference.

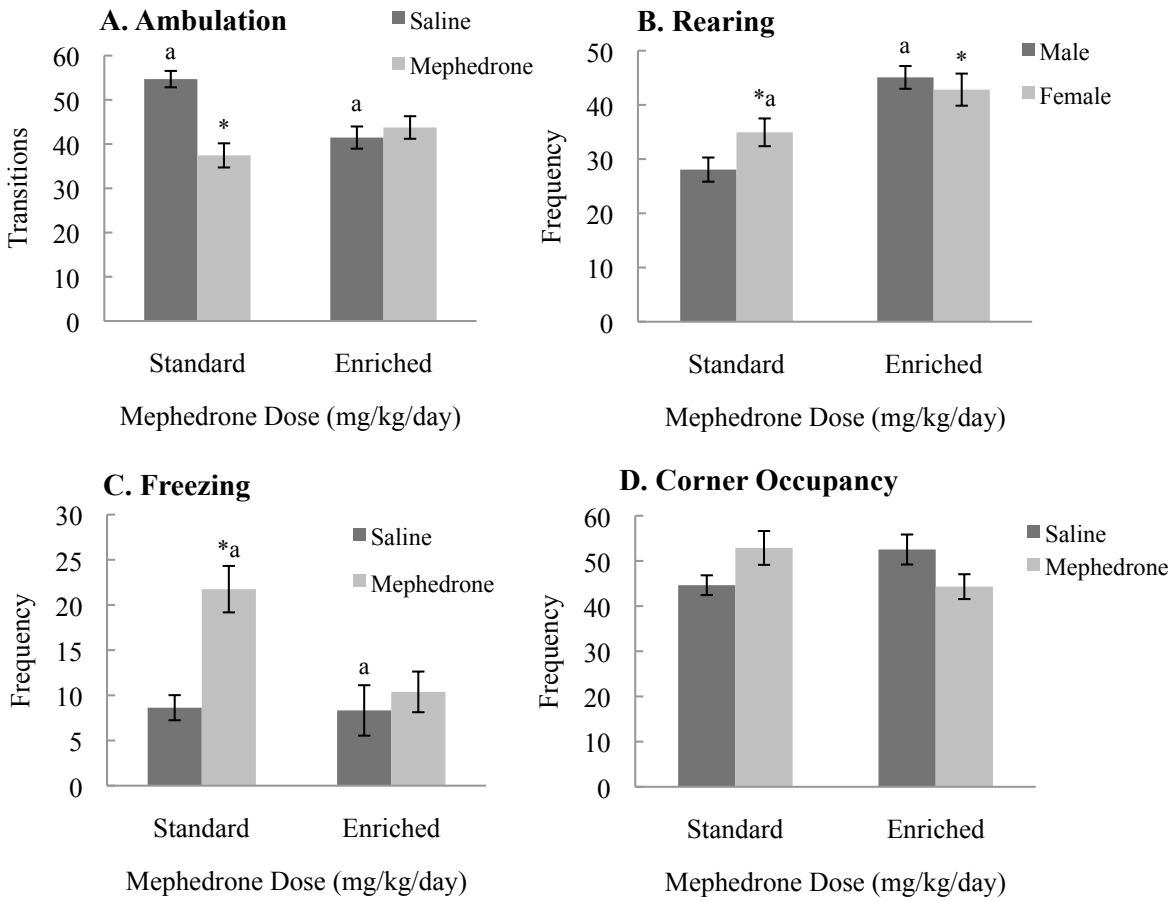

Figure 16. Mean ± S.E.M. frequencies for both standard and enriched caging conditions on (A) ambulation, (B) rearing, (C) freezing, and (D) corner occupancy in the open field. (A), (C), and (D) display results for both drug treatment and saline control groups (males and females combined). (B) displays results for males and females separately (drug treatment and control combined).
*Significantly different from control (A,C,&D) or males (B) for that caging condition. ($p < 0.05$).
[a,b,c]Groups with superscripts in common are significantly different ($p < 0.05$).

12.2 Light/Dark Box Results

The effects of mephedrone and enrichment on measures in the light/dark box are displayed in Table 6. As can be seen, mephedrone significantly decreased the number of entries into the light side. Mephedrone also appears to have increased first emergence latency and decreased time spent in the light side of the apparatus, though these differences are not statistically significant. Enrichment significantly increased observations in the light side, and appears to have decreased first emergence latency, while increasing the number of prior emergences (though these measures are also non-significant).

Table 6
Means, S.E.Ms, and ANOVA results for effects of Drug and Enrichment on all LD measures.

	Drug			Enrichment		
Measure	Saline	Mephedrone	F(1,55)	Standard	Enriched	F(1,55)
1st Emergence Latency (secs)	21.77(±2.52)	29.72(±6.29)	1.27	26.91(±4.70)	24.68(±5.10)	0.10
Entries Light Side	5.26(±0.33)	4.78(±0.29)	7.83**	5.34(±0.30)	5.42(±0.35)	0.42
Observations Light Side[a]	36.61(±2.34)	32.44(±2.22)	2.00	30.31(±1.97)	38.81(±2.38)	8.12**
Prior Emergences	0.71(±0.16)	0.75(±0.22)	0.01	0.47(±0.12)	1.00(±0.23)	3.99

*p < 0.05, **p < 0.01, ***p < 0.001
[a]Drug × Sex × Enrichment interaction significant, F(1,55) = 7.30, p < 0.01

As shown in Table 6 there was a significant drug × sex × enrichment interaction for the observations in the light side measure. This is displayed graphically in Figure 17. As can be seen; enriched, female subjects treated with mephedrone spent significantly less time in the light side of the light/dark box than saline controls but this effect was not true for enriched, male subjects (who showed no significant difference between drug and control groups).

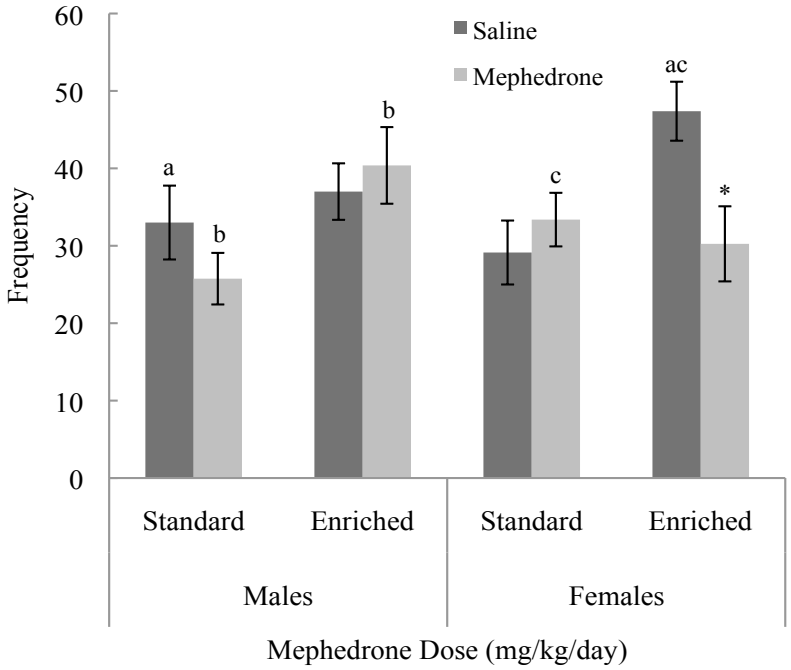

Figure 17. Mean ± S.E.M. frequencies for observations in the light side of the light/dark box. Data shown represents males and females, standard and enriched caging, and mephedrone and saline treatment conditions separately.
*Significantly different from saline control for that sex and caging condition. ($p < 0.05$).
[a,b,c]Groups with superscripts in common are significantly different ($p < 0.05$).

12.3 Elevated Plus Maze Results

The effects of mephedrone and enrichment on measures recorded in the elevated plus maze are displayed in Table 7. As can be seen, neither mephedrone treatment nor environmental enrichment had a significant impact on the percentage entries into open arms, or relative time spent in open arms of the maze. Mephedrone and enrichment both significantly reduced the number of entries into open arms.

Table 7
Means, S.E.Ms, and ANOVA results for effects of Drug and Enrichment on all EPM measures.

	Drug			Enrichment		
Measure	Saline	Mephedrone	F(1,55)	Standard	Enriched	F(1,55)
% Entries Open into Arms	0.52(±0.02)	0.50(±0.02)	0.29	0.50(±0.02)	0.51(±0.02)	0.09
% Observations in Open Arms	0.56(±0.02)	0.51(±0.03)	1.60	0.55(±0.03)	0.52(±0.03)	0.48
Entries into Closed Arms	7.39(±0.33)	5.41(±0.34)	17.56***	6.94(±0.36)	5.80(±0.37)	5.42*

*p < 0.05, **p < 0.01, ***p < 0.001

12.4 Sex Differences

The overall sex differences for each of the behavioural measures examined are presented in Table 8. As can be seen, only one measure reached statistical significance: females were more ambulatory than males in the open field. The other measures show smaller or no differences, though the general pattern mostly reflects female subjects performing more of a given behaviour than males.

Table 8
Means, S.E.Ms, and ANOVA results for main effects of Sex on all measures.

	Sex of Rats		
Measure	Male	Female	F(1,55)
Open Field			
Ambulation	40.87(±2.00)	47.78(±1.91)	9.47**
Walking	45.87(±1.48)	45.91(±1.48)	0.02
Rearing	36.29(±2.16)	38.88(±2.05)	0.91
Freezing	13.48(±1.86)	11.22(±1.89)	0.79
Grooming	4.35(±0.44)	4.00(±0.48)	0.33
Centre Occupancy	9.61(±1.17)	10.44(±0.92)	0.29
Corner Occupancy	47.90(±2.30)	49.13(±2.20)	0.13
Light/Dark Box			
1st Emergence Latency (secs)	32.25(±6.35)	20.53(±2.62)	2.27
Entries Light Side	5.26(±0.33)	5.50(±0.32)	0.26
Observations Light Side	33.94(±2.26)	35.03(±2.35)	0.11
Prior Emergences	0.84(±0.21)	0.63(±0.17)	0.68
Elevated Plus Maze			
% Entries in Open Arms	0.49(±0.02)	0.52(±0.02)	0.80
% Observations in Open Arms	0.53(±0.03)	0.54(±0.03)	0.05
Entries into Closed Arms	6.42(±0.41)	6.34(±0.35)	0.04

*p < 0.05; **p < 0.01; ***p < 0.001

12.5 Non-Behavioural Measures

The difference in weight (in grams) between treatment and testing ('adolescence' and 'adulthood' respectively) was calculated and compared across experimental conditions. Only sex had a significant effect on the weight gained during this period, with males gaining over 150% the weight of females. The mean ±SEM weight gain for each group were: saline = 82.40±3.88, mephedrone = 87.58 ±3.87, $F(1,55) = 1.98$, $p=0.16$; standard = 83.94 ±3.71, enriched = 86.16 ±4.09, $F(1,55) = 0.68$, $p=0.41$; and male = 102.90 ±2.73, female = 67.72 ±1.69, $F(1,55) = 116.30$, $p<0.001$.

13.0 Discussion

Experiment 3 examined the teratological properties of mephedrone on anxiety-related behaviours, and the potential of environmental enrichment to protect against these effects. Subjects were raised in either standard (i.e., 'impoverished') or enriched (containing toys or objects to stimulate activity and exploration) cages from weaning until behavioural testing took place in adulthood (approximately 4 months). During a developmental period analogous to mid-late adolescence in humans, subjects were treated with 20mg/kg of mephedrone (or saline) once per day, for ten consecutive days, representing a chronic 'binge-like' cycle. No subsequent manipulations were performed until the subjects reached adulthood, at which time they were tested for their degrees of anxiety-related behaviours in the open field, light/dark box, and elevated plus maze behavioural testing paradigms. The results generally supported both Hypothesis 1 (that subjects treated with mephedrone during adolescence would display more anxiety-related behaviours compared to control) and Hypothesis 2 (that subjects raised in enriched environments would display fewer anxiety-related behaviours than those raised in standard cages). There was also emerging support from one of the tests for Hypothesis 3: that environmental enrichment would mitigate the anxiogenic effects of mephedrone (relative to the standard caging condition).

13.1 Open Field Findings

13.1.1 Drug effects. Adolescent treatment with mephedrone produced a number of significant differences (relative to saline control) when the same subjects were later tested in the open field paradigm as adults (i.e., 4 months post-treatment). Ambulation was significantly decreased, as was the frequency of rearing behaviours. Conversely, freezing behaviours displayed a significant increase following mephedrone treatment. Although these differences are indicative of increased anxiety and support Hypothesis 1, they are more appropriately considered in the light of significant interactions discussed below. Increased

anxiety is consistent with similar research showing a lasting anxiogenic effect of adolescent treatment with other designer drugs, namely benzylpiperazine and MDMA (Aitchison & Hughes, 2006; Kolyaduke & Hughes, 2013).

The remaining open field measures (walking, grooming, centre occupancy and corner occupancy) did not show significant differences between saline and mephedrone groups, though a visual inspection of the results shows these data to also be in the directions predicted by the hypotheses. No firm conclusions can be drawn from these particular measures, though it is possible that they reflect an anxiogenic effect of mephedrone (Hypothesis 1) that is present but too weak to produce significant differences (i.e., perhaps the behavioural teratological effect of acute mephedrone administration diminishes over time following cessation of drug administration).

13.1.2 Enrichment effects. Those subjects raised in enriched environments displayed significantly less walking and freezing behaviours, and more rearing behaviours in the open field. These results are indicative of reduced anxiety and support Hypothesis 2. The remaining measures were non-significant, though as with the results described above, several of the measures were in the predicted direction (e.g., center and corner occupancy were suggestive of reduced anxiety).

13.1.3 Interaction effects. Some interesting drug × enrichment effects were noted in the open field results. Mephedrone significantly decreased ambulation and increased freezing behaviours (results indicating more anxiety, as predicted by Hypothesis 1) for subjects raised in standard cages but not for those raised in enriched cages. Similarly, although there were no significant main effects of mephedrone on the corner occupancy measure, the pattern of responding was different for those subjects raised in standard cages versus enriched cages. As can be seen visually in Figure 16D, for subjects raised in standard cages, mephedrone produced more corner occupancy than the control group (though this difference was not

significant), whereas for subjects raised in enriched cages this effect was reversed. These findings tend to support Hypothesis 3 and suggest that environmental enrichment may confer some degree of resilience or resistance against the anxiogenic properties of mephedrone.

A sex × enrichment interaction was also significant in the open field results. It was found that for standard cages, female subjects reared significantly more often (a behaviour indicative of less anxiety) than male subjects. In enriched cages however, there was no significant difference in rearing behaviours between male and female subjects (though both sexes reared more than in standard cages). This finding may suggest that the benefits of environmental enrichment are different for males than for females; perhaps that males have more to gain from this manipulation than females.

13.2 Light/Dark Box Findings

13.2.1 Drug effects. Following adolescent treatment with either mephedrone or saline there were a number of effects and patterns noted in adulthood using the light/dark box testing paradigm. Subjects treated with mephedrone made significantly fewer entries into the light side of the light/dark box (a behaviour indicative of increased anxiety) compared to control subjects. While there were no significant differences found for first emergence latency or for time spent in the light side, a visual inspection of the results shows these measures to be in the predicted directions as well. Similar to findings in the open field, these measures may reflect a degree of anxiety-like behaviour that has lessened over time (since drug administration) so that now it is not potent enough to be detected using the current methodology. While no firm conclusions can be currently drawn from these measures alone, taken together with the number of entries measure, and the open field results, these findings suggest support for Hypothesis 1 in that those subjects who were treated with mephedrone were more anxious than those in the control group. This conclusion is consistent with previous research into drug effects measured in the light/dark box (Bourin & Hascoet, 2003).

The prior/partial entries measure was included as it was thought that it may represent a degree of risk assessment behaviour (Arrant et al., 2013). The current results found no difference between mephedrone or control groups, suggesting that either this measure (as utilised by the current methodology) does not accurately measure risk assessment behaviours, or that adolescent mephedrone use does not produce a lasting effect on risk assessment behaviours in adults. It is therefore concluded that the latter explanation is more likely to apply as significant differences in this response were noted in Experiment 1 that examined the acute effects of mephedrone. It may be that these acute effects on risk assessment behaviours fade with the acute drug intoxication, or perhaps that risk assessment is fundamentally different in adolescents than in adults, so the expression of the effect is dependent on the developmental time of exposure. This would be worth exploring in future research.

13.2.2 Enrichment effects. Subjects raised in enriched environments took less time to fully emerge into the light side of the apparatus, made more entries of the light side, and spent significantly more time in the light side compared to those raised in standard cages. However, only the difference for the latter response was statistically significant. These results are suggestive of reduced anxiety in the enriched condition (supporting Hypothesis 2), which adds further evidence to previous research that has found environmental enrichment to have an anxiogenic effect on rodents (Benaroya-Milshtein et al., 2004; Laviola et al., 2004).

Environmental enrichment nearly doubled the occurrence of prior/partial entrances into the light side, suggesting that this type of enriched environment may confer an increase in cautionary or risk assessment behaviours in subjects. While the noted effect did not reach statistical significance, it was very close to achieving significance ($p = 0.0507$) and may have suffered from inadequate statistical power owing to the small frequencies being observed.

Something interesting appears to be occurring here, and future research may need to adapt the current methodology in order to better observe any effect.

13.2.3 Interaction effects. An interesting drug × sex × enrichment effect was noted for the observations in the light side measure. For female subjects; those raised in standard cages showed no significant effect of mephedrone treatment, whereas those subjects raised in enriched cages showed a significant decrease in time spent in the light side following treatment with mephedrone. For males, this drug × enrichment effect was not found; instead, in both standard and enriched cages mephedrone had no significant effect on observations in the light side. These results suggest that enrichment mitigated the anxiogenic effect of mephedrone (which supports Hypothesis 3) for females but not for males. Combined with the sex × enrichment interaction noted in the open field results, this is further evidence that the ability of environmental enrichment to mitigate the anxiogenic effects of mephedrone may be different for males and females.

13.3 Elevated Plus Maze Findings

13.3.1 Drug effects. Adolescent treatment with mephedrone did not have any significant effects on the anxiety-related measures of the elevated plus maze when subjects were tested in adulthood. The only measure that displayed a significant difference was ‘entries of the closed arms’, a control measure that was added to assess levels of general locomotor activity uncontaminated by anxiety (Cruz et al., 1994). Adolescent treatment with mephedrone caused a significant decrease in the number of entries into the closed arms relative to the control group. Taken together, these findings indicate that the subjects treated with mephedrone were generally less ambulatory in the elevated plus maze than those who received saline, though neither groups showed any preference for either the closed arms or the open arms (which would suggest more or less anxiety respectively).

These findings do not support the hypotheses, and are inconsistent with the results we would expect based on similar studies in the literature (Hughes et al., 2015; Pellow et al., 1985; Silva & Brandao, 2000). However, the current findings are consistent with the results observed in Experiment 1, which also found no significant differences on any measures except for entries into the closed arms. If the acute exposure to mephedrone did not produce significant anxiogenic effects as assessed by this test, then logically such effects would be unlikely to appear when tested later in life. Experiment 2 however, did find anxiogenic effects of mephedrone in the elevated plus maze and at the same dose used in this study (20mg/kg). The acute effects observed in Experiment 2 may not have produced lasting teratological effects observable in the current study, though the results of the open field and light/dark box tests discussed earlier would refute this. Few conclusions can be drawn from the elevated plus maze results, as the findings appear to be inconsistent. It is unclear why this is the case, so addressing this issue will be an important facet of any future research.

13.3.2 Enrichment effects. As with the drug effects discussed above, only the 'entries into the closed arms' measure displayed any significant effects of enrichment. Environmental enrichment significantly reduced the number of entries into the closed arms of the elevated plus maze, indicating that those subjects raised in enriched environments were generally less ambulatory in the elevated plus maze than those raised in standard cages.

13.4 Sex Differences

As with the findings of Experiments 1 and 2, female subjects generally engaged in more locomotor and exploratory behaviours than male subjects, in keeping with sex differences already acknowledged in previous reports (Kokras & Dalla, 2014). The only measure to produce a significant difference however (besides the interaction effects already discussed), was ambulation in the open field, where females were significantly more ambulatory than males (again consistent with previous research).

13.5 Non-Behavioural Measures

Weight was the only non-behavioural measure recorded in this study, with subjects being weighed both during treatment (i.e., 'adolescence') and again at testing (i.e., adulthood). The difference in weight between these two times was calculated, and the only significant variable affecting this measure was sex: male subjects gained significantly more weight than females. Enrichment did not significantly affect weight gain, nor did drug treatment. This latter point may be significant in telling us that chronic mephedrone use during adolescence may not impair *physical* growth and development, although this one measure is insufficient to justify any more than hypothesis formulation at present.

13.6 Limitations and Future Research

A number of limitations and ideas for future research have already been discussed in this section: the inclusion of a prior/partial emergences measure in the light/dark box may represent risk assessment behaviour following acute drug treatment, but the utility and validity of this measure when examining chronic or teratological effects remains to be explored; the inconsistent findings in the elevated plus maze need to be adequately explained, and subsequently remedied (if possible); and weight gain between adolescence and adulthood, alone, is not sufficient to generalise any conclusions about physical development.

A potential methodological issue with this study, when viewed in the wider context of the three Experiments combined, is that in the time between Experiments 2 and 3 the animal facility changed the food available to the rats in their home cages. This would seem an insignificant change except that the researcher and the animal technicians noted that the animals in Experiment 3 appeared smaller than usual. This suspicion was confirmed when historical data from a similar experiment conducted in the same lab in 2012 showed that the average weight of a subject on the first day of adolescent treatment (PND45) then was 100 grams, compared to 68 grams in the current study (average weights on the final day of

treatment were 130 grams and 85 grams respectively). The animals otherwise appeared to be healthy and developing at a normal rate. It is unclear what impact this might have had, if any, on the interpretability of the current findings, though the majority of the results suggest that this was not a significant confounding variable as statistical differences in the predicted ranges were still found.

It would be interesting to determine if the behavioural teratological effects of mephedrone that were observed in this study are permanent, or if they might fade over time, i.e., if the animals were re-tested after another year would the effect still be seen, and if so, still as severe? Similarly, would the positive effects conferred by environmental enrichment remain if the enrichment were removed? Or if enrichment were only introduced after the adolescent mephedrone treatment, could the anxiogenic effects be reversed post-hoc? There are a number of ways, and combinations of these two variables that remain to be explored.

A number of findings in this study suggest that environmental enrichment's ability to mitigate the anxiogenic effects of adolescent mephedrone use (i.e., Hypothesis 3) differs for males and females. This is an interesting discovery that warrants further investigation of why this is so, what mechanisms are involved, and what practical implications this may have for designing interventions that are effective both for males and for females.

13.7 Conclusion

The results from both the open field and light/dark box tests support Hypothesis 1 (that chronic mephedrone use during adolescence would result in more anxiety-like behaviours when tested later in adulthood), and Hypothesis 2 (that environmental enrichment would reduce the frequency of anxiety-like behaviours). A number of significant interactions also supported Hypothesis 3 (that environmental enrichment would mitigate the anxiogenic effects of mephedrone use during adolescence), though interestingly this effect was found to affect males and females differently. It appears that mephedrone can be a significant

behavioural teratogen when used during adolescence, producing changes in behaviour that are long-lasting, if not permanent. This has significant implications for public health as adolescence is typically the age when people are most likely to experiment with recreational drugs (National Instutute on Drug Abuse, 2014; Spear, 2007). The finding that environmental enrichment is capable of reducing or preventing this long-term harm is an exciting prospect that may inform how such resilience, and perhaps recovery, could be conferred on people who fall victim to these drugs of abuse.

14.0 Summary and Conclusion

In the ever-changing world of designer drugs, governments, healthcare providers, and even researchers seem to constantly be one step behind the drug makers. Users buy these drugs, often under the impression that they are safe alternatives to known drugs of abuse, or at least without any explicit knowledge that they are *un*safe alternatives. Likewise, many legislative bodies are seemingly powerless to act on these ever-emerging new designer drugs until evidence begins to pile up showing a harmful effect. In many ways, the well-established drugs of abuse such as methamphetamine, cocaine, and MDMA, may be considered the safer choices in light of the fact that their effects (horrific as they can be) are at least comparatively well-documented and understood. When the effects of these drugs are known, this can inform policy-makers (who can attempt to control the supply and availability of dangerous drugs), healthcare providers (who can better respond to the medical and psychological needs of drug users), and consumers (who may elect not to use a drug that has been shown to be unsafe). Given the speed and relative ease with which new drugs are constantly being created, it can appear to be an 'uphill battle' for drug researchers to understand each new compound, but the potential for harm resulting from inaction makes the pursuit of such information a vitally important and noble goal.

For all of the reasons described above, the current study sought to increase our collective knowledge about the effects of mephedrone; one of the primary compounds representing the present generation of designer drugs; the synthetic cathinone derivatives, popularly known as 'bath salts'. Despite first being synthesised nearly 90 years ago, mephedrone has only become a popular drug of abuse in the last few years, prompting a need for its harmful sequelae to be better known. Research in the last few years has expanded our understanding of the pharmacology and toxicology of mephedrone, and we have learned that in many ways this drug is analogous to amphetamine. A gap in our current knowledge is

arguably that few studies have been conducted specifically examining the *behavioural* sequelae of mephedrone use. This was the rationale for the current study, which used animal models to examine the effects of mephedrone on anxiety-related behaviours in three randomised controlled experiments.

In Experiment 1, 50 male and 50 female rats were administered mephedrone at doses of 0 (saline control group), 1, 20, 40, and 60 mg/kg, and then the anxiety-like behaviours of the subjects were measured in the open field, light/dark box, elevated plus maze, and novel object recognition behavioural testing paradigms. The results of Experiment 1 indicated that mephedrone had an anxiogenic effect, and that this effect was generally dose-dependent. Interestingly, the dose-response curve was different for males and females, with males displaying the predicted inverted 'U' shaped curve (anxiety increased with higher doses of mephedrone up until 20mg/kg, then decreased), whereas females instead displayed a linear increase (as mephedrone dose increased, so too did anxiety-related behaviours. It was hypothesised that this drug × sex interaction was due to differences in the expression of stereotypy behaviours induced by mephedrone. This was subsequently explored in Experiment 2.

Experiment 2 followed a similar methodology to Experiment 1 (using mephedrone doses of 0, 1, 10, and 20 mg/kg) to examine anxiety-related behaviours in the open field and elevated plus maze, and additionally included some measures designed to assess stereotypy behaviours. The results again showed mephedrone to produce a dose-dependent anxiogenic effect, and also a dose-dependent effect on stereotypy (the higher the dose of mephedrone, the greater the frequency of stereotypy behaviours observed). As predicted, these stereotypy behaviours were expressed differently for males and females, with males typically displaying more non-ambulatory stereotypies (such as head swaying), and females, more movement-based stereotypies (such as running around in circles ['gyration']). These sex differences in

the *expression* of behaviours (as opposed to the degree to which the behaviours are performed), make it very difficult, if not impossible, for any firm conclusions to be drawn regarding sex differences in the underlying concept (e.g., "Are males more anxious than females, overall?"). The researcher noted that stereotypy, as a potential confound to anxiety-related measures, may be inextricably connected to the observable effects of anxiety, and as such requires consideration in the interpretation of results from these studies (and others like them).

Experiment 3 looked at the potential for mephedrone to cause lasting effects on anxiety-related behaviours when used chronically during the developmental period of adolescence. The results showed that following chronic, adolescent use of mephedrone, more anxiety-like behaviours were still evident later in life when compared to a saline control group. This suggests a behavioural teratological effect of mephedrone that can persist beyond acute cessation. As the secondary aim of the experiment, it was also observed that the noted effect on anxiety-related behaviours was largely mitigated by environmental enrichment, though interestingly, while both males and females enjoyed this increased 'resilience', enrichment conferred its benefits differently for both sexes. It is unclear from this study why males and females would differ in their accrued advantages following environmental enrichment, though realising that the two sexes have such different responses to this intervention may prove vital to any practical applications that may result from such early research.

The primary aims of the study were accomplished over the three experiments, but overall, perhaps the most impressive and interesting findings have been those of the sex differences and drug × sex interaction effects. The results of this study add further support to a growing and compelling movement within the animal research field to including both male and female subjects in research designs. If either males or females were not included in the

current study, many of the most important and useful findings would have been missed, and the conclusions less generalisable.

The findings of these three experiments raise some interesting implications for the human users of mephedrone. That these drugs can cause anxiety and related symptoms has been established before in case reports and some survey-based research, whereas now we have some evidence to suggest that the dose of mephedrone ingested can dependently increase the magnitude of the given effect, and that it may do so differently for men and for women. The sex differences and drug × sex interactions found in the current study may be some of the more important findings in terms of influencing our understanding of mephedrone's effects in humans; the results suggest that while men and woman likely both experience a dose-dependent effect of mephedrone (generally speaking, higher doses produce more of a given effect), the overt expression of the effects may differ between the sexes, with females tending towards more active, movement-based behaviours compared to males. Practically speaking, this may translate to men displaying more internalising behaviours (such as depression, psychosis, and paranoia), whereas women may display more externalising behaviours (such as hyperactivity, and self-mutilation). Based on the current findings, females also appear to be more sensitive to the hyperthermic effects of mephedrone, which suggests an increased risk and susceptibility (compared to males) to some of the more dangerous physiological sequelae of mephedrone use.

Another interesting implication is that of the enrichment findings from Experiment 3. That environmental enrichment was able to mitigate some of the negative consequences of chronic, adolescent, mephedrone use, is an exciting and promising feature that may lead to tangible therapeutic solutions for treating drug abuse disorders in humans. More research is needed here though to discover how environmental enrichment confers its benefits (i.e., what are the mechanisms of action within the brain), and under what conditions (i.e., does prior

enrichment protect against later drug exposure, or does post-hoc enrichment reverse the subsequent effects of exposure?).

The findings of these three experiments should be viewed as a beginning to our understanding of the behavioural effects of mephedrone use; there are many areas left still to be explored, and many explanations that remain yet to be found. An obvious limitation of the current study is the lack of any neurochemical assays, biological analyses, or investigations into potential mechanisms of action for the observed behavioural effects. In many ways the current study has opened more avenues for exploration than it has closed, and it was because of this nearly limitless scope that the researcher chose to confine the investigation exclusively to behavioural analyses. Given the present findings, and some of the questions subsequently raised, it would be interesting for future research to examine the neuro-biological aspects of the current results, in order to provide a more balanced view, and to potentially infer some causative mechanisms.

It could be argued that the real 'war on drugs' is not being fought by law enforcement officials on the front line, but by drug researchers in their laboratories. Currently the only defence we have against the ever-increasing waves of new designer drugs is information: the more we know about what we are facing the better equipped we are to deal with the threat that they pose to individuals and to society. With new synthetic drugs being created all the time, it may seem like a losing and costly battle to research every new compound; but it must be remembered, amongst all the science, that these drugs have a very real effect on real human lives, so the cost of not researching them will always be much higher.

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Appendix A

Animal Ethics Approval for Experiment 1

UNIVERSITY OF CANTERBURY
Te Whare Wānanga o Waitaha
CHRISTCHURCH NEW ZEALAND

ANIMAL ETHICS COMMITTEE

Secretary, Lynda Griffioen
Email: animal-ethics@canterbury.ac.nz

Ref: 2013/26R

18 November 2013

Rikki Thompson
Department of Psychology
UNIVERSITY OF CANTERBURY

Dear Rikki

I am pleased to inform you that the Animal Ethics Committee (AEC) has approved your application entitled: "The behavioural effects and psychopharmacology of Methylmethcathinone (Mephedrone) or "bath salts" on hooded rats"

Approval has been granted:
(a) for the use of 100 Rattus norvegicus
(b) for your research project to be undertaken from 18 November 2013 to 30 June 2014. If you require an extension of this period please contact the AEC Secretary.

As part of AEC's new Code of Ethical Conduct all applicants receiving approval to work on animals are required to provide a final report at the completion of their project. The purpose is to provide the AEC with a record of your use of animals and what was achieved by your research project. We are very much interested in your findings and to learn what you have achieved. Following the completion date indicated above you are asked to provide this report using the new Final Report form which is available at the AEC web site (https://intranet.canterbury.ac.nz/research/ethics.shtml).

On an annual basis the University is legally required to provide to MAF statistical data on all animal manipulations undertaken in a calendar year. To assist us in collating this information you are also required to complete and return to the AEC Secretary the attached MAF Animal Manipulation Statistical form 30 days after the completion of this project, or once every three years, which ever comes first. If no animals have been manipulated in your project please provide a "Nil" return. Please also find enclosed a copy of the Animal Welfare (Records and Statistics) Regulations 1999 for your information, together with a list of Animal Type Codes and brief guideline notes for your assistance.

Yours sincerely

Associate Professor Jim Briskie
Chair
University of Canterbury Animal Ethics Committee

Appendix B

Open Field Record Sheet

Rat ID:_____ Adolescent drug condition:_____ Rat sex:_____ Date:_____
Enrichment condition: **Open-field behaviour**

1)				
2)				
3)				
4)				
5)				
6)				
7)				
8)				
9)				
10)				
11)				
12)				
13)				
14)				
15)				
16)				
17)				
18)				
19)				
20)	40)	60)	80)	100)

Transitions = _____ Centre squares (6, 7, 10, 11) = _____

Corner squares (1, 4, 13, 16) = _____ Rearing (R) = _____

Walking (W) = _____ Grooming (G) = _____

Freezing (F) = _____ # faecal boluses = _____

Transitions 1-60 = _____ *Transitions 61-100* = _____

Appendix C

Elevated Plus Maze Record Sheet

Rat/ID_____ Group_____ Sex_____

Elevated plus maze

Occupying arms 1, 2, 3, 4 (Specify which are the **OPEN** and which are the **CLOSED** arms)

1)					
2)					
3)					
4)					
5)					
6)					
7)					
8)					
9)					
10)			***2.5 min***		
11)					
12)					
13)					
14)					
15)					
16)					
17)					
18)					
19)					
20) ***1 min***		40) ***2 min***	60) ***3 min***	80) ***4 min***	100) ***5 min***

Observations: arm 1 =_____ arm 2 =_____ arm 3 =_____ arm 4 =_____:

TOTAL **OPEN** arms = _____; TOTAL **CLOSED** arms = _____;

Entries arm 1_____

Entries arm 2_____

Entries arm 3_____

Entries arm 4_____

TOTAL entries **OPEN** arms= _____; TOTAL entries **CLOSED** arms = _____;

TOTAL **BOTH** = _____

Appendix D

Light/Dark Box Record Sheet

Light-dark box behaviour

Rat ID:_____ Adolescent drug condition:_____ Rat sex:_____ Date:_____

Enrichment condition:_____

1)				
2)				
3)				
4)				
5)				
6)				
7)				
8)				
9)				
10)				
11)				
12)				
13)				
14)				
15)				
16)				
17)				
18)				
19)				
20)	40)	60)	80)	100)

1st emergence latency (in seconds) = _____

Partial entries into LIGHT side = _____

Entries of LIGHT side = _____

Observations/100 in LIGHT side = _____

faecal boluses = _____

Appendix E

Novel Object Recognition Record Sheet

Novel object recognition: Rat/ID_____ Group_____ Sex_____
[Proximity: <2cm from object. Novel object = N, familiar object = F]

	1st min		2nd min		3rd min		4th min		5th min	
	Proximity from N or F object	Contact with N or F object	Proximity from N or F object	Contact with N or F object	Proximity from N or F object	Contact with N or F object	Proximity from N or F object	Contact with N or F object	Proximity from N or F object	Contact with N or F object
1										
2										
3										
4										
5										
6										
7										
8										
9										
10										
11										
12										
13										
14										
15										
16										
17										
18										
19										
20										
TOT										

Appendix F

Photos of observed adverse effects following mephedrone treatment.

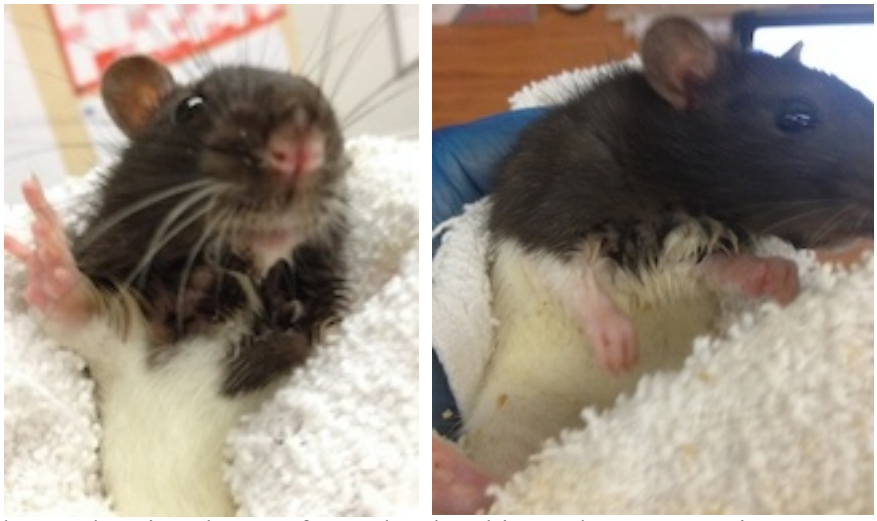


Figure F1. Photos showing the wet fur under the chin or ‘heat prostration’

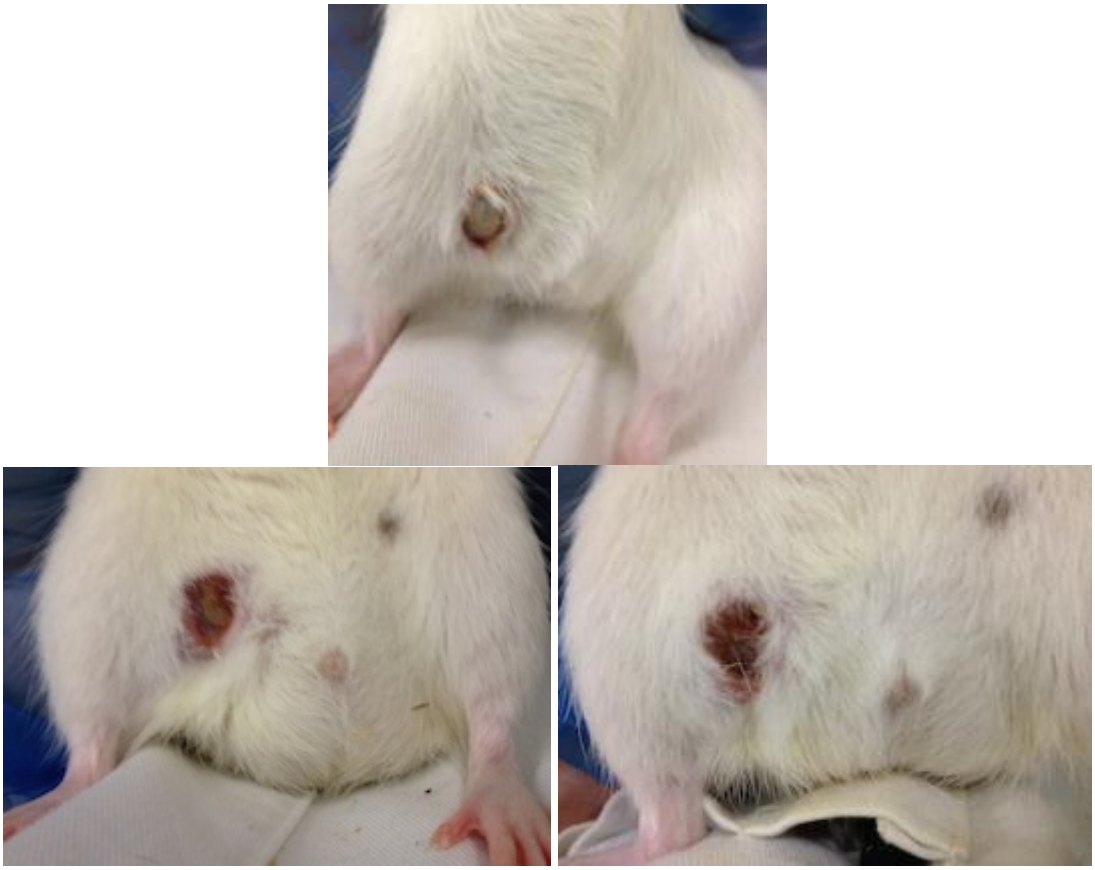


Figure F2. Photos showing the abscesses/sores at the injection sites on some animals.

Appendix G

Animal Ethics Approval for Experiment 2

UNIVERSITY OF CANTERBURY
Te Whare Wānanga o Waitaha
CHRISTCHURCH NEW ZEALAND

ANIMAL ETHICS COMMITTEE

Secretary, Lynda Griffioen
Email: animal-ethics@canterbury.ac.nz

Ref: 2014/24R

17 November 2014

Rikki Thompson
Department of Psychology
UNIVERSITY OF CANTERBURY

Dear Rikki

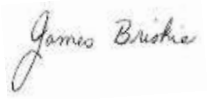
I am pleased to inform you that the Animal Ethics Committee (AEC) has approved your application entitled: "The behavioural effects and psychopharmacology of Methylmethcathinone (Mephedrone) or "bath salts" on hooded rats".

Approval has been granted:
(a) for the use of 64 Rattus norvegicus
(b) for your research project to be undertaken from 1 December 2014 to 1 March 2015. If you require an extension of this period please contact the AEC Secretary.

As part of AEC's new Code of Ethical Conduct all applicants receiving approval to work on animals are required to provide a final report at the completion of their project. The purpose is to provide the AEC with a record of your use of animals and what was achieved by your research project. We are very much interested in your findings and to learn what you have achieved. Following the completion date indicated above you are asked to provide this report using the new Final Report form which is available at the AEC web site (https://intranet.canterbury.ac.nz/research/ethics.shtml).

On an annual basis the University is legally required to provide to MPI statistical data on all animal manipulations undertaken in a calendar year. To assist us in collating this information you are also required to complete and return to the AEC Secretary the attached MPI Animal Manipulation Statistical form 30 days after the completion of this project, or once every three years, whichever comes first. If no animals have been manipulated in your project please provide a "Nil" return. Please also find enclosed a copy of the Animal Welfare (Records and Statistics) Regulations 1999 for your information, together with a list of Animal Type Codes and brief guideline notes for your assistance.

Yours sincerely



Associate Professor Jim Briskie
Chair
University of Canterbury Animal Ethics Committee

Appendix H

Animal Ethics Approval for Experiment 3

UNIVERSITY OF CANTERBURY
Te Whare Wānanga o Waitaha
CHRISTCHURCH NEW ZEALAND

ANIMAL ETHICS COMMITTEE

Secretary, Lynda Griffioen
Email: animal-ethics@canterbury.ac.nz

Ref: 2015/08R

25 May 2015

Rikki Thompson
Department of Psychology
UNIVERSITY OF CANTERBURY

Dear Rikki

I am pleased to inform you that the Animal Ethics Committee (AEC) has approved your application entitled: "The behavioural effects and psychopharmacology of methylmethcathinone (mephedrone) or "bath salts" on hooded rats".

Approval has been granted:
(a) for the use of 64 *Rattus norvegicus*
(b) for your research project to be undertaken from 25 May 2015 to 30 September 2015. If you require an extension of this period please contact the AEC Secretary.

As part of AEC's new Code of Ethical Conduct all applicants receiving approval to work on animals are required to provide a final report at the completion of their project. The purpose is to provide the AEC with a record of your use of animals and what was achieved by your research project. We are very much interested in your findings and to learn what you have achieved. Following the completion date indicated above you are asked to provide this report using the new Final Report form which is available at the AEC web site (https://intranet.canterbury.ac.nz/research/ethics.shtml).

On an annual basis the University is legally required to provide to MPI statistical data on all animal manipulations undertaken in a calendar year. To assist us in collating this information you are also required to complete and return to the AEC Secretary the attached MPI Animal Manipulation Statistical form 30 days after the completion of this project, or once every three years, whichever comes first. If no animals have been manipulated in your project please provide a "Nil" return. Please also find enclosed a copy of the Animal Welfare (Records and Statistics) Regulations 1999 for your information, together with a list of Animal Type Codes and brief guideline notes for your assistance.

Yours sincerely

James Briskie

Associate Professor Jim Briskie
Chair
University of Canterbury Animal Ethics Committee

Appendix I

Photos from enriched and standard caging conditions.

Figure I1. Examples of toys/objects used in the cages of the enrichment condition.

Figure I2. Example of an "impoverished" cage from the standard condition.